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The Journal of Laboratory and Clinical Medicine

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SEVERE SCURVY

A Clinical and Hematologic Study

Richard W. Vilter, M.D., Robert M. Woolford, M.D., and Tom D. Spies, M.D.
Cincinnati, Ohio

DURING the past few years we have studied nineteen patients seriously ill with scurvy, many of whom had severe anemia. This report deals primarily with the morphologic characteristics of the blood and the clinical and hematologic responses to synthetic vitamin C therapy which were observed in these subjects. Certain characteristics of the anemia which have not been described previously suggest that hemolysis may be an etiologic factor. The number of patients in this series and the severity of their disease emphasize that bachelor scurvy remains a medical and public health problem in large municipal hospitals.

MATERIAL AND METHODS

From January, 1935, to June, 1945, nineteen patients with a primary diagnosis of scurvy have been admitted to the Medical Service of the Cincinnati General Hospital.

Eleven of these patients, all of whom were admitted during 1942, 1943, 1944, and 1945, have been under our direct observation and are the subjects of the hematologic study. The eight additional patients (Table I, Cases 1 to 8) were studied only through data obtained from their hospital records. Since these eight individuals were treated in many different ways, only the hematologic observations made on admission and some of the distinctive clinical features will be reported.

On each of the eleven patients (Table II, Cases 9 to 19) detailed medical and neurologic histories were obtained and complete physical and neurologic examinations performed. Diet histories were obtained and analyzed* whenever the patients could give accurate accounts of their food habits. Red and white blood cell counts† were done on peripheral blood with pipettes and counting chambers certified by the United States Bureau of Standards. Hemoglobin was determined as oxyhemoglobin with the Evelyn photoelectric colorimeter.[1] Hematocrit determinations were made on oxalated venous blood (4 mg. potassium oxalate and 6 mg. ammonium oxalate per 5 c.c. of blood) centrifuged for thirty minutes in a Wintrobe tube at 3,000 r.p.m. Reticulocyte and platelet counts were made by the wet technique using Dameshek's method.[2] Cover slip preparations for cytologic study of capillary blood and sternal marrow were stained with Wright-Giemsa stains. The bone marrow specimens were obtained by sternal aspiration.

The amount of ascorbic acid in the plasma was determined in eleven patients (Cases 9 to 19) by the method of Farmer and Abt.[3] Icteric indices, qualitative van den Bergh reactions, foam, iodine, and fuming nitric acid tests for bile in the urine and the dilution

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*The authors are indebted to Mrs. Vinton Siler, Miss Helen Volk, Miss Carol Jones, Mrs. Katherine Shafer Irwin, Miss Lillian Lanzi, Miss June Hamlin, and Miss Roberta Thomas, Dietetic Department, Cincinnati General Hospital, for analyses of the diets and for dietary supervision of the patients on the vitamin C restricted diet.

†All hematologic studies were performed by us assisted by Mrs. Nancy Lavery.

TABLE I. INITIAL EXAMINATION OF

CASE	PATIENT	SEX	AGE	YEAR	R.B.C. (MILLIONS)	HB. (GM.)	RETICULOCYTES (%)	PLATELETS	HEMATOCRIT
1	J. B.	M	75	1937	2.66	9.5	5.0	—	23
2	M. C.	M	31	1943	3.45	9.0	—	—	—
3	M. J.	F	59	1940	2.64	10.4	—	—	—
4	G. F.	M	54	1938	2.08	6.4	7.5	380,000	18
5	S. J.	M	42	1936	4.03	14.4	—	—	—
6	F. E.	M	35	1938	4.35	13.5	—	210,000	40
7	H. W.	M	32	1942	3.44	9.2	6.0	316,000	29
8	W. J.	M	16	1942	2.90	8.8	—	—	—

This data has been gathered from Cincinnati General Hospital charts.

method[4] using Ehrlich's aldehyde reagent for urobilinogen in twenty-four hour urine specimens were performed in thirteen patients (Cases 1, 4, and 9 to 19) and were repeated in seven (Cases 11 and 14 to 19) every other day until they were normal. The amount of urobilinogen in the stool was determined for three patients (Cases 17 to 19) by Watson's method.[5]

Prothrombin times were estimated in eleven patients (Cases 4, 9, 10 and 12 to 19) by Quick's method.[6] Total serum proteins were determined by the Kjeldahl technique in eight of the subjects (Cases 4, 10 to 13, 15, 17, and 19) and albumin-globulin ratios were determined in four (Cases 4, 10, 12 and 13). The cephalin flocculation test according to the method of Hanger[7] and the bromsulfalein excretion test using 2 mg. dye per kilogram of body weight and the blood sample drawn thirty minutes after the injection of the dye were carried out in seven patients (Cases 11 and 14 to 19). Determinations of serum iron[8] were performed* on three patients (Cases 17 to 19).

The diet used in this study will be called the vitamin C restricted diet. For each twenty-four hour period, it provided the following foods: a glass of boiled milk, one egg or less, cottage cheese, American cheese, polished rice, a thoroughly cooked vegetable made into purée, mashed potato, noodles, stewed dried fruit or apple sauce, syrup, crackers, jam, and coffee. The diet contained no meat, citrus fruits, tomatoes, cabbage, lettuce, or uncooked vegetables and provided about one-third of the minimum daily requirement of the B complex vitamins. It provided approximately 12 mg. of iron daily and several milligrams of vitamin C as a maximum.

Case summaries for those patients whose hematologic courses are presented in graphic form accompany their respective figures.

CLINICAL AND LABORATORY MANIFESTATIONS BEFORE THERAPY WITH VITAMIN C

Occurrence of Scurvy.—Of the nineteen patients studied, five were admitted to the Cincinnati General Hospital between 1935 and 1941, three in 1942, three in 1943, five in 1944, and three during the first six months of 1945 (see Fig. 1). The majority were admitted in the spring or early autumn months (see Fig. 2). The patients ranged in age from 16 to 84 years; twelve were older than 50 years. Eighteen subjects were men; one was a woman.

Predisposing Factors.—Thirteen of the nineteen patients were bachelors or had been widowers for more than a year. They cooked their own meals or ate alone in restaurants. In several instances, citrus fruits were too expensive for their limited budgets, but for the most part these individuals just did not

*The authors are indebted to Dr. Carl V. Moore and to Miss Virginia Minnich, M.S., Washington University, St. Louis, Mo., for the determination of these serum iron values.

PERIPHERAL BLOOD ON EIGHT PATIENTS

M.C.V.	M.C.H.	M.C.H.C.	W.B.C.	DIFFERENTIAL W.B.C.				
				SEGMENTED NEUTROPHILES	LYMPHOCYTES	MONOCYTES	EOSINOPHILES	BASOPHILES
83	35	41	5,600	78	16	4	2	0
—	—	—	5,900	78	20	2	0	0
—	—	—	11,200	85	14	1	0	0
90	32	35	7,150	77	19	4	0	0
—	—	—	3,750	61	33	4	0	2
			13,800	82	15	3	0	0
84	29	32	6,650	58	35	4	2	1
—	—	—	3,700	58	23	19	0	0

take the trouble to procure oranges, grapefruit, or tomatoes. Relatives, when there were any, were too busy in industry or in the Army or Navy to see that the elderly unproductive members of the family had adequate food.

Of the remaining six patients, scurvy developed because of the following reasons: poor dietary selection in two who were mentally deficient (Cases 2 and 6); paregoric addiction and restricted food intake in one (the only female patient, Case 3); chronic alcoholic addiction and dietary insufficiency in one (Case 12); and self-prescription of vitamin C deficient diets for dyspepsia in two (Cases 5 and 7).

In addition, the following factors were probably of some importance. Two patients had spastic hemiparesis (Cases 9 and 11), the result of old cerebrovascular accidents, and one had residual signs of Jamaica ginger paralysis (Case 17). These chronic neurologic disorders limited the ability of the subjects to obtain food. One patient had minimal active pulmonary tuberculosis (Case 2), and six had chronic bronchitis and emphysema (Cases 1, 3, 9, 11, 13, and 15). These infections may have increased their vitamin requirements.

Because of one or more of these reasons, none of the patients had had fresh citrus fruit for six months or more prior to admission.

Clinical Signs of Vitamin C Deficiency.—It was difficult to date the exact time of onset of the clinical disease. All of the patients complained of fatigue, weakness, and anorexia for months or years and had noted bruises usually related to slight trauma for a few weeks or several years before admission. Most of the patients said that their present illness began one or two months before admission with the onset of dull aching in the legs and swelling and pain in the knee or ankle joints. These symptoms were followed by the appearance of purplish discolorations about the affected joints, extending up and down the legs and arms and occasionally over the abdomen and face. Later, the subjects with natural teeth noted painful, swollen gums which interfered with mastication and from which blood oozed. With the onset of these major symptoms, the severity of the dyspnea, weakness, dizziness, lethargy, and anorexia increased, and many patients were aware that the color of their urine became dark red.

The most characteristic clinical sign was the minute perifollicular hemorrhage (see Plate I). It was found in sixteen subjects (Cases 2, 4 to 10, and 12 to 19), usually on the extensor surfaces of the legs or arms.

TABLE II. INITIAL EXAMINATION OF PERIPHERAL BLOOD ON ELEVEN PATIENTS WITH SCURVY CARRIED OUT IN HEMATOLOGIC LABORATORY OF CINCINNATI GENERAL HOSPITAL

CASE	PATIENT	SEX	AGE	YEAR	R.B.C. (MILLIONS	HB. (GM.)	RETICULOCYTES (%)	PLATELETS	HEMATOCRIT	M.C.V.	M.C.H.	M.C.H.	W.B.C.	DIFFERENTIAL W.B.C.				
														SEGMENTED NEUTROPHILES	LYMPHOCYTES	MONOCYTES	EOSINOPHILES	BASOPHILES
9	J. M.	M	84	1943	2.68	9.5	6.6	205,920	29.5	110	35	32	10,500	64	27	8	1	0
10	J. K.	M	63	1943	3.03	10.6	5.2	178,500	31	102	35	34	3,300	82	11	6	1	0
11	W. B.	M	73	1944	1.74	5.8	1.0	205,320	17	98	33	34	4,450	79	17	2	2	0
12	L. F.	M	45	1942	2.18	6.3	5.0	120,000	21	97	29	30	12,600	76	14	10	0	0
13	A. S.	M	46	1944	2.25	5.7	3.5	157,000	18.5	82	25	31	2,700	81	8	10	0	0
14	C. K.	M	68	1944	2.81	9.2	8.4	252,900	30	107	33	31	5,400	69	20	8	2	1
15	J. H.	M	63	1944	3.30	9.7	1.0	303,600	30	91	29	32	9,750	80	14	6	0	0
16	C. G.	M	75	1944	1.88	7.8	10.1	475,000	21.5	115	41	36	3,750	64	33	3	0	0
17	H. E.	M	57	1945	2.73	8.5	5.0	—	26	95	31	33	5,000	60	30	7	3	0
18	C. M.	M	75	1945	2.46	7.5	5.7	270,000	26	105	30	29	5,200	72	15	10	2	1
19	T. K.	M	66	1945	2.46	7.0	8.2	408,000	23	94	28	30	5,500	52	39	8	1	0

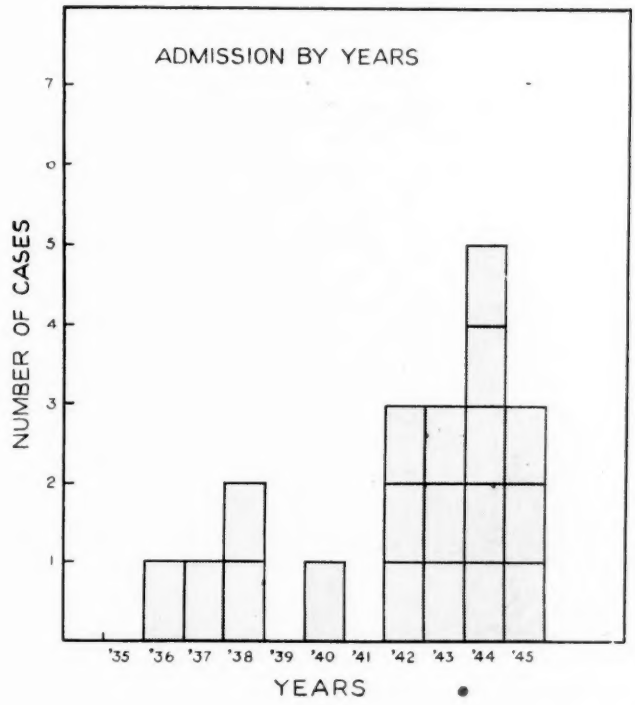
ADMISSION BY YEARS
NUMBER OF CASES
1
2
3
4
5
6
7
'35 '36 '37 '38 '39 '40 '41 '42 '43 '44 '45
YEARS

Fig. 1.

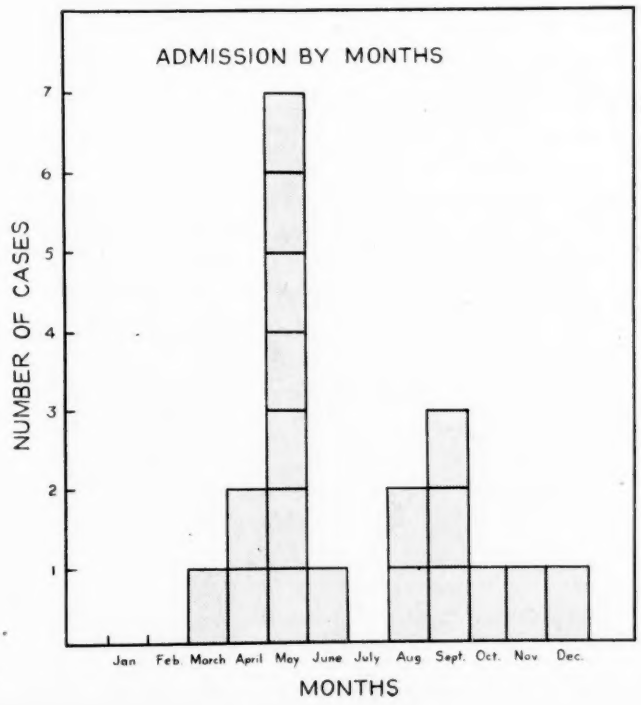
ADMISSION BY MONTHS
NUMBER OF CASES
1
2
3
4
5
6
7
Jan Feb. March April May June July Aug Sept. Oct. Nov. Dec.
MONTHS

Fig. 2.

Seventeen patients (Cases 1, 3, 4, and 6 to 19) had purpura and ecchymoses ranging from a few millimeters to 20 cm. in diameter (Plate II, *A* and *B*). These ecchymoses were most common around the knees, ankles, and wrists and in the popliteal spaces. In many instances they occurred along superficial scratches and abrasions of the skin. Thromboses of saphenous, popliteal, femoral, or cephalic veins often were associated with the larger ecchymoses.

Four patients (Cases 11, 14, 15, and 19) had deep subcutaneous or intramuscular hemorrhages—hard, tender, deep masses, stretching the overlying skin of the arm or leg. Nine subjects (Cases 1, 4 to 8, 10, 11, and 15) had tender knee and ankle joints; five (Cases 1, 4, 6, 7, and 10) had moderately swollen joints; and in one (Case 5) the knee joint capsule was so tense with fluid that aspiration was done. Thirty cubic centimeters of grossly bloody fluid were removed.

Acute gum lesions were found in thirteen (Cases 2, 4 to 10, 12 to 15, and 19) of the fourteen patients (see Plate III, *A* and *B*) who had teeth or remnants thereof. No gum lesions occurred in those who were edentulous. The interdental papillae and gingival margins were greatly swollen, blue-red, tender, and highly friable. In some patients the swelling was so great that the teeth were nearly covered by gum tissue (Plate IV, *A*). The color changes were distinctive, even when the subject had a hemoglobin level of 6 or 7 Gm. The breath usually was foul, and the gums oozed blood following slight trauma; however in no patient was there spontaneous hemorrhage or blood loss in excess of a few cubic centimeters daily.

The patients generally had sallow, dirty grayish-yellow cadaveric complexions. Their hands and feet were cyanotic and cold, and in five subjects (Cases 5, 13, 14, 18, and 19) long thin splinter hemorrhages were observed under the nails (Plate IV, *B*). The sclerae were slightly icteric, and the ankles were edematous.

Twelve patients (Cases 2 to 4, 6, 7, 10 to 13, 15, 16, and 19) had temperature elevations ranging between 99.6 and 102° F., pulse rates ranging from 90 to 120 beats per minute, and respirations from 24 to 32 per minute. Blood pressures were normal or low in all except three subjects (Cases 1, 11, and 19) whose blood pressures were 195/100, 170/100, and 190/100, respectively, on admission and 155/80, 140/80, and 140/90 six days later just before they received antiscorbutic medication.

Associated Vitamin Deficiency Diseases.—Since the diets of most of these individuals were deficient in more than one essential nutrient, evidence of vitamin deficiency diseases other than scurvy was common.

Seven patients (Cases 2 to 4, 10, 12, 15 and 19) had mild peripheral neuritis of thiamine deficiency; four (Cases 4, 10, 12 and 18) had fiery red glossitis of niacin deficiency; and two (Cases 3 and 16) had the magenta-colored glossitis and cheilosis of riboflavin deficiency. Five patients (Cases 2, 5, 8, 13, and 16) had follicular hyperkeratoses of the arms, legs, and buttocks, interpreted as evidence of vitamin A deficiency, and one (Case 8) had a healed rachitic chest deformity.

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Plate I.

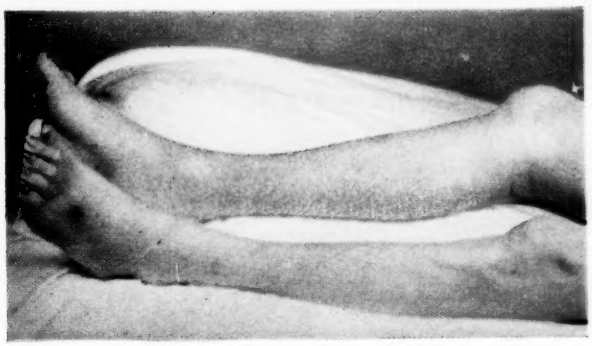

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Plate II.

Plate I.—Patient 8. Perifollicular hemorrhages.

Plate II.—Patient 16. *A*, Massive scorbutic ecchymoses. *B*, Same area as *A* after sixteen days of therapy with vitamin C.

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A. Plate III. B.

A. Plate IV. B.

Plate III.—Patient 9. *A*, Blue-red swollen gum about the remains of a tooth. *B*, Same gum area shown in *A* after twenty days of vitamin C therapy.

Plate IV.—Patient 2. *A*, Swollen spongy necrotic gums in a Negro with scurvy. *B*, Splinter hemorrhages in a patient with scurvy. These hemorrhages are similar to those observed in Patient 14.

TABLE III. BILE PIGMENT STUDIES, LIVER FUNCTION TESTS, AND SERUM IRON DETERMINATIONS

CASE	PATIENT	ICTERIC INDEX	QUALITATIVE VAN DEN BERGH REACTION	URINE UROBILINOGEN (DILUTION)	STOOL UROBILINOGEN (MG./24 HR.)	URINE BILE	PROTHROMBIN TIME (SEC.)	SERUM PROTEIN (GM./100 C.C.)	SERUM ALBUMIN (GM./100 C.C.)	SERUM GLOBULIN (GM./100 C.C.)	CEPHALIN FLOCCULATION (IN 48 HR.)	BROMSULFALEIN (% IN ½ HR.)	SERUM IRON (MG./%)
1	J. B.	20				0							
4	G. F.	12	Indirect	+		0	22 (15)*	6.0	3.2	2.8			
9	J. M.	12	Indirect	1/16		0	18 (15)						
10	J. K.	12				0	15 (15)	5.18	2.43	2.75			
11	W. B.	22	Biphasic† and indirect	1/1024		0		5.98			+++	10	
12	L. F.	10	Biphasic† and indirect			0	18.5 (14)	5.5	2.9	2.6			
13	A. S.	12	Indirect	1/32		0	15 (15)	4.3	2.6	1.7			
14	C. K.	16	Biphasic† and indirect	1/64		0	20 (17)				++	<5	
15	J. H.	20	Biphasic† and indirect	1/32		0	16.5 (17)	6.68			+	<5	
16	C. G.	15	Indirect	1/128		0	17 (17)				+	<5	
17	H. E.	10	Biphasic† and indirect	1/16	336	0	15 (15)	6.5			+	<5	0.116
18	C. N.	14	Biphasic† and indirect	1/64	897	0	16 (15)				+	15	0.083
19	T. K.	13	Biphasic† and indirect	1/32	945	0	14 (15)	6.47			+	15	0.072

*Normal value for the day.
†Reaction delayed beyond five minutes.

TABLE IV. DIFFERENTIAL BONE MARROW COUNTS

CASE	PATIENT	POLYMORPHONUCLEAR LEUCOCYTES (%)	METAMYELOCYTES (%)	MYELOCYTES C (%)	MYELOCYTES B (%)	MYELOCYTES A (%)	MYELOBLASTS (%)	LYMPHOCYTES (%)	MONOCYTES (%)
9	J. M.	47.5	30.5	14.5	1.0			2.5	0.5
10	J. K.	34.5	24.5	21.0	4.5	1.5	1.5	1.5	1.0
11	W. B.	39.5	32.5	9.0	1.0	1.0	1.0	8.5	0.5
12	L. F.	32.5	36.5	15.5	2.0	2.5		1.5	0.5
13	A. S.	49.0	22.5	11.5	2.5			0.5	
14	C. K.	53.0	26.0	11.0	1.5			1.0	
15	J. H.	43.5	32.5	6.5	1.5			4.5	
16	C. G.	42.0	39.0	9.0	1.0		2.0	2.0	
17	H. E.	42.0	21.0	13.5	8.0	1.0	0.5	4.5	0.5
18	C. N.	41.5	22.5	13.5	5.0	0.5	0.5	3.5	0.5
19	T. K.	38.5	26.0	19.5	1.0	0.5		5.5	

TABLE V. BONE MARROW OF PATIENTS WITH SCURVY

CASE	PATIENT		POLYMORPHONUCLEAR LEUCOCYTES (%)	METAMYELOCYTES (%)	MYELOCYTES C (%)	MYELOCYTES B (%)	MYELOCYTES A (%)	MYELOBLASTS (%)	LYMPHOCYTES (%)
15	J. H.	Before vitamin C	43.5	32.5	6.5	1.5			4.5
		Vitamin C 10 days	47.5	25.0	15.0	0.5			3.0
19	T. K.	Before vitamin C	38.5	26.0	19.5	1.0	0.5		5.5
		Vitamin C 7 days	54.5	23.5	17.0	1.5	0.5	0.5	3.5
11	W. B.	Before vitamin C	39.5	32.5	9.0	1.0	1.0	1.0	8.5
		Vitamin C 20 days	55.5	23.5	14.5	1.0			2.0
16	C. G.	Before vitamin C	42.0	39.0	9.0	1.0		2.0	2.0
		Vitamin C 4 days	37.5	34.5	15.0				4.0
		Vitamin C 9 days	39.5	39.0	13.0	1.0			1.5
		Vitamin C 25 days	53.0	28.5	7.5	2.5		0.5	5.0

Analyses of the diets of thirteen subjects revealed that in three instances (Cases 4, 6, and 17) only vitamin C was grossly deficient; in five (Cases 10, 13 to 16) the vitamins of the B complex, vitamin C, and protein were grossly inadequate; and in the remaining five all essential nutrients were presumably eaten in inadequate amounts.

Laboratory Data.—No gross or occult blood was found in the urine, stool, or vomitus. Albumin was present in moderate amounts in the admission urine specimens of eight patients but cleared rapidly after bed rest was instituted. Icteric indices ranged from 10 to 22. The van den Bergh reactions were of the indirect or delayed biphasic type, and the urine contained an excess of urobilinogen but no bile. Stool urobilinogen content for twenty-four hours ranged from 336 to 945 mg. (normal, from 100 to 200 mg.). The prothrombin times were normal in seven of the eleven patients tested. Total serum proteins ranged from 4.31 to 6.68 Gm. per 100 c.c. The albumin values varied from 2.43 to 3.2 Gm. Cephalin flocculation tests were considered normal except in one

ON ELEVEN PATIENTS WITH ANEMIA OF SCURVY

EOSINOPHILES (%)	EOSINOPHILIC MYELOCYTES (%)	BASOPHILES (%)	PLASMA CELLS (%)	HEMOCYTOBLASTS (%)	MEGALOBLASTS PER 100 W.B.C.	EARLY ERYTHROBLASTS PER 100 W.B.C.	LATE ERYTHROBLASTS PER 100 W.B.C.	NORMOBLASTS PER 100 W.B.C.	NUMBER OF NUCLEATED R.B.C. PER 100 W.B.C.
2.0	1.0		0.5		1.0	2.5	22.0	152.5	178.0
2.0	0.5		6.0		0.5	1.5	5.5	59.5	67.0
1.5			4.0	1.5	1.5	7.5	30.0	41.5	80.5
2.5			6.0		0.5	2.5	6.5	68.5	78.0
3.0	3.0	0.5	5.5	2.0		0.5	2.5	85.5	88.5
3.5			3.5	0.5		1.0	9.5	136.5	147.0
1.5	1.0	0.5	8.5		1.0	2.5	11.0	69.5	84.0
2.0		1.0					8.0	76.5	84.5
2.5	1.5	1.5	3.5		1.5	10.0	31.5	139.5	182.5
5.0	1.0	0.5	6.0			2.5	5.5	63.5	71.5
5.0	0.5		3.5		1.5	4.5	22.5	75.0	103.5

BEFORE AND AFTER ADMINISTRATION OF VITAMIN C

MONOCYTES (%)	EOSINOPHILES (%)	EOSINOPHILIC MYELOCYTES (%)	BASOPHILES (%)	PLASMA CELLS (%)	HEMOCYTOBLASTS (%)	MEGALOBLASTS PER 100 W.B.C.	EARLY ERYTHROBLASTS PER 100 W.B.C.	LATE ERYTHROBLASTS PER 100 W.B.C.	NORMOBLAST PER 100 W.B.C.	NUMBER OF NUCLEATED R.B.C. PER 100 W.B.C.
	1.5	1.0	0.5	8.5		1.0	2.5	11.0	69.5	84.0
0.5	1.5	1.5	0.5	5.0		0.5	4.5	32.5	223.0	261.5
	5.0	0.5		3.5		1.5	4.5	22.5	75.0	103.0
	0.5	1.5	0.5	2.0	0.5	1.5	8.0	18.5	167.0	195.0
0.5	1.5			4.0	1.5	2.5	15.0	60.0	83.5	161.0
0.5		1.5	0.5	0.5	0.5		1.0	2.5	59.0	62.5
	2.0		1.0	2.0				8.0	76.5	84.5
	1.0	0.5	1.0	6.5			1.0	8.5	56.5	66.0
	0.5	2.5	0.5	2.5			1.0	7.0	52.0	60.0
	1.5	2.5	1.0	2.5				1.0	25.0	26.0

patient (Case 11); bromsulfalein retention at thirty minutes varied from 5 to 15 per cent (Table III). Gastric analyses showed hypochlorhydria or achlorhydria after histamine stimulation. The plasma vitamin C content was 0.0 mg. per cent. Values for serum iron were normal; they ranged from .072 to 0.116 mg. per cent.

Hematologic Data.—The peripheral blood characteristics for all patients are summarized in Tables I and II. Two patients with mild scurvy of recent onset (Cases 5 and 6) had erythrocyte counts over 3.5 million and hemoglobin values over 12 Gm. The erythrocyte counts in the seventeen more severely ill subjects ranged from 1.74 to 3.44 million and the hemoglobin levels, from 5.8 to 10.5 Gm. The cells were normochromic and normocytic or moderately macrocytic; they varied only slightly in size and shape. The admission white cell counts were under 6,000 in twelve of the nineteen patients. Some were as low as 2,500. Differential white cell counts were normal. Platelets occurred in normal or moderate reduced numbers. Initial reticulocyte counts ranged from

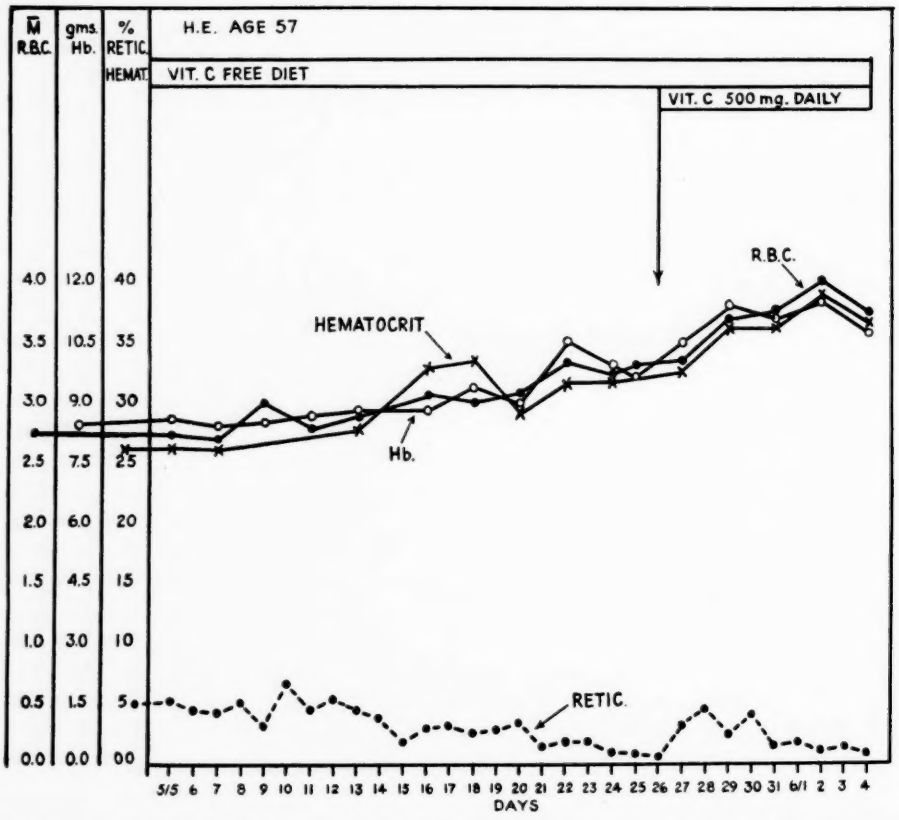

Fig. 3.—Illustrating the gradual hematologic recovery of a patient with moderately severe scurvy of short duration, following rest in bed. Administration of vitamin C appears to have been associated with a small increase in reticulocytes and an acceleration of erythrocyte and hemoglobin regeneration.

H. E., (Case 17), a 57-year-old white man, was admitted to the Cincinnati General Hospital May 5, 1945. For one week prior to admission he had had pain in bones and joints, swelling of the left elbow and left knee, and painful swelling of the left thigh with purplish discoloration of the skin overlying this area. Similar swelling and pain in the left upper extremity had occurred several days prior to admission. Small red-blue spots also had appeared on the arms and legs, and he had become weak and dizzy.

He had had Jamaica ginger paralysis in 1930. Since this time he had been living alone, moving from bed to chair, and cooking for himself. His diet consisted of two meals a day. For breakfast he had two eggs, a bowl of cereal, and four pieces of bread. Lunch consisted of one quart of milk, graham crackers, and whole wheat bread. He never ate citrus fruits and very seldom ate green vegetables. He attempted to make up for this lack of vitamins by taking Hemo.

The temperature was 98° F.; pulse, 100; blood pressure, 140/90. There were extensive ecchymoses over the left popliteal and left anticubital areas, extending up and down the left leg and left arm. Phlebothrombosis was present in both areas, and considerable collateral venous circulation was observed over the left shoulder. There was fluid in the left knee. Small areas of purpura and perifollicular hemorrhages were noted over the arms and legs. There were splinter hemorrhages under the nails. Teeth were absent, and gums were essentially normal. Neurologic examination showed a spastic quadriplegia with muscle atrophy

(Continued on opposite page.)

3 to 10 per cent except in two patients (Cases 11 and 15) in whom initial counts of 1 per cent were obtained.

The bone marrow appeared to be moderately hypercellular in five patients (Cases 9, 10, 11, 13, and 19), normally cellular in five (Cases 12, 14, 15, 17, and 18), and moderately hypocellular in one subject (Case 16). Differential counts of nucleated cells showed a relative increase in erythrocyte progenitors with the majority of these cells at the normoblast and late erythroblast stages of development (Table IV). In one patient (Case 11) who had the most severe anemia we have encountered, 11 per cent of the young red cells were megaloblasts and early erythroblasts. In all marrow specimens, the granulocyte series was cytologically normal or showed a slight shift to young cell forms.

CLINICAL COURSE AND RESPONSE TO THERAPY

The eleven patients under our direct observation were kept on the vitamin C restricted diet for the period of hospitalization and were given placebos until their safety seemed jeopardized or until improvement occurred from bed rest alone. The shortest period of preliminary observation was two days; the longest, twenty-one days.

Two patients (Cases 17 and 18) showed slow but definite clinical and hematologic improvement on bed rest alone. On admission they were classed as mildly or moderately ill. Even though their plasma vitamin C remained 0.0 mg. per cent, their perifollicular hemorrhages and ecchymoses cleared over a period of from fourteen to twenty-one days, and they felt subjectively improved. Icteric indices and urine urobilinogen concentrations reached normal values in the same period of time. In one subject (Case 18) the erythrocytes and hemoglobin had increased 500,000 cells and 2.4 Gm., respectively, at the end of twelve days, and reticulocytes had fallen from 5.7 to 1.6 per cent. Administration of vitamin C at this time did not seem to accelerate blood formation. One subject (Case 17) showed much slower blood regeneration. During a period of twenty-one days, the erythrocyte count increased from 2.75 to 3.27 million and the hemoglobin from 8.5 to 9.6 Gm. Reticulocytes fell from 5.0 to 0.8 per cent. Bone marrow aspiration repeated at this time showed

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of the extremities. The calves were tender, and there was diminished perception of light touch and vibration over the legs and feet. No jaundice was noted and the skin color was normal.

For data concerning blood examinations, liver function tests and bile pigment studies, and bone marrow examinations, see Tables II, III, and IV. Bleeding time was two and one-half minutes; clotting time, six minutes. Plasma ascorbic acid was 0.0 mg. per cent; blood urea nitrogen, 14 mg. per cent.

The patient was placed on the vitamin C restricted diet and was observed for twenty-one days. The lesions of scurvy slowly cleared, and the erythrocyte count and hemoglobin level gradually rose from 2.73 to 3.23 million and from 8.5 to 9.8 Gm., respectively. Plasma vitamin C level was still 0.0 mg. per cent.

Administration of vitamin C, 100 mg. five times a day by mouth, was begun May 26, 1945. A very slight elevation of reticulocytes occurred and possibly some increase in the rate of erythrocyte regeneration.

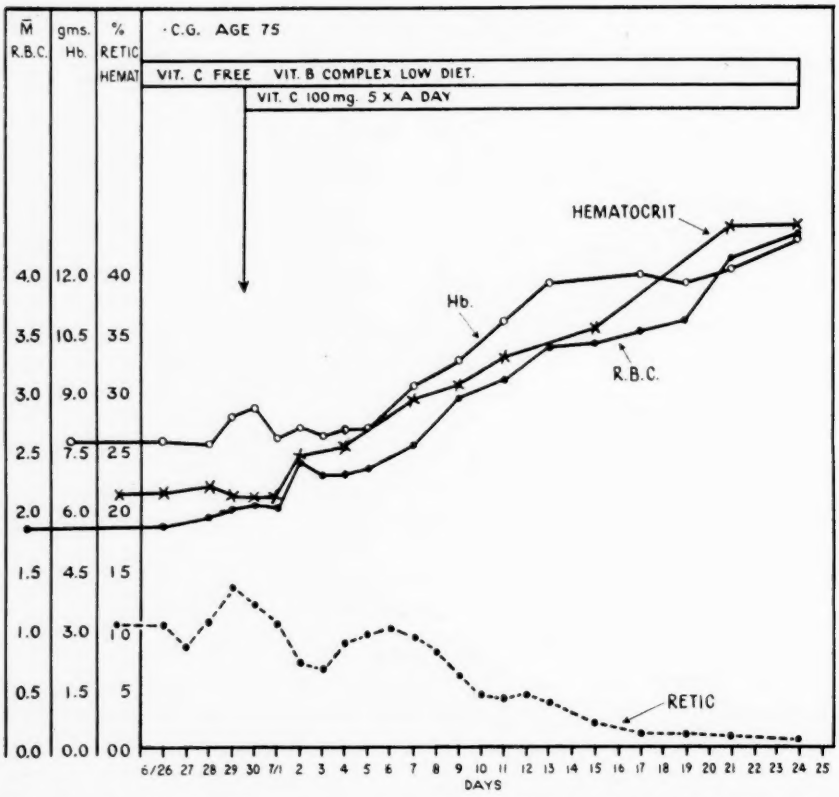

Fig. 4.—A patient with severe scurvy. The erythrocyte count and hemoglobin were unchanged before vitamin C therapy, but the patient's physical condition deteriorated so rapidly that vitamin C had to be administered as a life-saving measure. Thereafter, the erythrocytes and hemoglobin regenerated rapidly as the reticulocytes decreased.

C. G. (Case 16), a 75-year-old white man, was admitted to the Cincinnati General Hospital June 26, 1944, because of weakness, aching legs, and purplish discoloration of the skin for three months. His wife had died one year before admission, and he had had no citrus fruit or tomatoes since that time. He prepared his own meals. Breakfast consisted of two eggs, bacon, and coffee. For lunch he ate soup, beans, and crackers. He had a sandwich and sometimes nothing for supper.

The temperature was 101° F.; pulse, 80; respirations, 24; blood pressure, 110/50. He was a lethargic old man, and the skin was a dirty lead gray color. There were perifollicular hemorrhages on the arms. The entire right leg to the crest of the ilium was covered by confluent ecchymoses. The left leg was less severely involved. The sclerae were icteric. He was edentulous and the tongue was smooth, fissured, and pale. The only abnormality on neurologic examination was absence of the knee and ankle jerks bilaterally.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. No vitamin C was found in the plasma.

The patient was placed on the control diet. By the fourth hospital day the blood pressure had fallen to 95/50 and he had become very lethargic and confused. Administration of 500 mg. of vitamin C intramuscularly in divided doses was begun. One day thereafter the temperature was normal, blood pressure, 115/55, and the patient was much less confused. Rapid improvement continued even though he developed cheilosis and a magenta tongue which responded satisfactorily to riboflavin. The icteric index reached normal in twelve days, and the erythrocyte count and hemoglobin reached 4.3 million and 12.9 Gm., respectively, in twenty-six days. Blood pressure at discharge was 130/70.

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depressed activity of all marrow elements. After vitamin C was administered, a small rise in reticulocytes occurred, and erythrocyte and hemoglobin regeneration appeared to be accelerated (see Fig. 3). Complete recovery did not occur until the subject was given an adequate diet.

Nine patients (Cases 9 to 16 and 19) classed on admission as severely ill did not show any clinical improvement during the observation period. The ecchymoses and perifollicular hemorrhages changed from dark reddish-purple to dark brownish-purple but did not fade or spread. Gingival lesions remained unchanged or progressed. Blood pressures, frequently at a low level on admission, fell. Icteric indices and urine urobilinogen concentrations rose. Two of the patients developed Cheyne-Stokes type of respiration. Mental torpor and cyanosis deepened, and the cadaveric gray skin color became more prominent. Six of these subjects remained unchanged hematologically (Cases 9 to 13 and 16) during the control period (see Figs. 4 and 5). Three patients (Cases 14, 15, and 19) had a rapid decline in erythrocytes and hemoglobin and an increase in reticulocytes as their clinical condition grew worse (see Figs. 6 and 7). When signs of impending shock became alarming, vitamin C was given in doses of 100 mg. five times a day, orally or intramuscularly, depending on the clinical condition of the patient.

All of these nine patitents improved within from twenty-four to forty-eight hours after administration of vitamin C. Nail beds became pink, the cadaveric gray color of the skin disappeared, the blood pressure rose, and the Cheyne-Stokes type of respiration was replaced by normal breathing. Those patients who had temperature and pulse elevations before the administration of vitamin C had normal temperatures and pulse rates within from one to three days afterward.

Perifollicular hemorrhages disappeared after from two to three days leaving the affected hair follicles hyperkeratotic and pigmented. The engorged, blue-red swollen gums began to clear after forty-eight hours. As the swelling receded, the gums retracted from the teeth and appeared pale, scarred, pitted, and thickened. Infected pockets between teeth and gum never healed completely. Joint swelling and pain disappeared over a period of from three to five days, and major ecchymoses disappeared over a period of from two to three weeks leaving large areas of pigmentation in their wake. Icteric indices and urine urobilinogen concentrations fell to normal levels in four days (Cases 14 and 15), six days (Case 19), and twelve days (Cases 11 and 16). Four days after vitamin C therapy the stool urobilinogen content had decreased from 945 to 265 mg. per 24 hours in the one subject (Case 19) in whom the test was repeated.

In two of these nine subjects (Cases 11 and 12) reticulocytes rose from 2 to 19 per cent and from 5 to 10 per cent by the tenth and sixth days, respectively (see Fig. 8). In seven patients the high reticulocyte levels were maintained or rose slightly between the fourth and seventh days, subsequently falling slowly to normal.

Red cell and hemoglobin increases began from four to seven days after vitamin C administration, and after three weeks the levels had reached 3.5

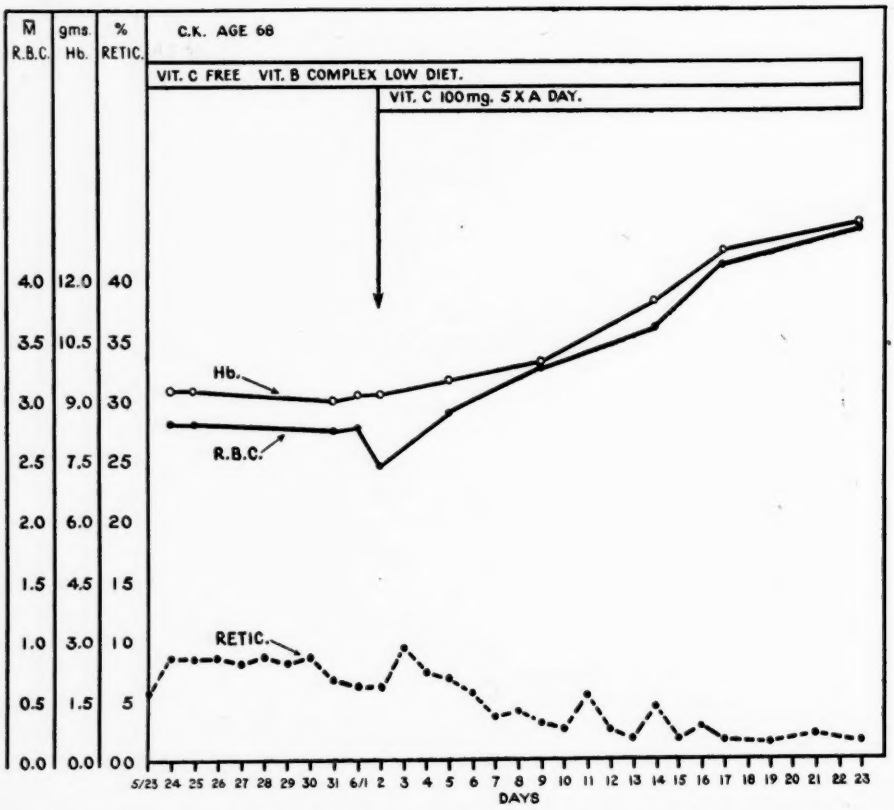

Fig. 5.—The course of a patient with severe scurvy on the vitamin C free diet. The erythrocytes and hemoglobin values were stationary but his clinical condition grew worse during the eleven days prior to the administration of vitamin C. Thereafter, he made a rapid clinical and hematologic recovery.

C. K. (Case 14), a 68-year-old single white man, was admitted May 22, 1944, because of intermittent leg and joint pain and sore gums of several months' duration. Six weeks before admission the right knee swelled and the popliteal area became ecchymotic. Pin-point purplish spots over the arms came and went during this period. The gums had been sore intermittently, and the teeth had been falling out for seven years. In 1943 he had been on the vascular service for phlebothrombosis and had been treated with vitamin C because of purpura over both lower extremities. His diet consisted of sausage meat, bread, coffee, and potatoes. He said he had never eaten fruit or green vegetables.

The temperature was 99° F.; pulse, 70; respirations, 24; and blood pressure, 94/55. The mouth contained one loose tooth snag surrounded by elevated, soft blue-red hemorrhagic mucous membrane. Otherwise the oral mucous membrane was normal. The tongue was smooth, pale, and clean. There were many small punctate hemorrhages on the dorsal surfaces of the arm and legs. There was a massive ecchymosis associated with phlebothrombosis behind the right knee. The skin was a sallow lead gray color. There were long, old splinter hemorrhages under the fingernails. The sclerae were icteric. The right leg was edematous.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. Gastric analysis showed 32° free acid after histamine stimulation. Bleeding time was one and one-half minutes; coagulation time, three and one-half minutes. Tourniquet test was negative after five minutes. No vitamin C was found in the blood plasma. Electrocardiogram showed low voltage upright T waves in Leads II and III; T_1 was diphasic; T_4, inverted.

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million and 12.5 Gm. or more in all patients. Repeated bone marrow aspirations on four subjects (Cases 11, 15, 16, and 19) after vitamin C was administered for four or more days showed reversion to normal marrow. In two instances a striking increase in the per cent of normoblasts and late erythroblasts (Table V) was found one week after the administration of vitamin C.

Of the eight persons whose hospital records were reviewed, one (Case 8) whose illness was mild showed complete healing of gum lesions, disappearance of perifollicular hemorrhages, and rapid rise in red blood cell count and hemoglobin on bed rest without benefit of crystalline vitamin C or citrus fruits. The other patients received vitamin C in doses of from 300 to 1,000 mg. per day after varying periods of study, during which vitamin C was restricted in the diet. Before vitamin C was administered, the blood pressure of one patient (Case 4) had fallen to 60/20. He developed signs of thrombosis of a branch of the right middle cerebral artery. Except for the residual hemiparesis, he made an uneventful recovery. Another subject (Case 1) received vitamin C only after he had experienced an episode of severe dyspnea and cyanosis associated with a precipitous fall in blood pressure. In spite of heroic efforts, he died six hours afterward in a similar episode. Autopsy was consistent with scurvy and revealed no other cause of death. The rest of the patients made complete and uneventful recoveries in the manner previously described.

DISCUSSION

The clinical observations recorded in this group of patients are similar to those previously reported,[9-11] but several features seem worthy of special emphasis. The general appearance of patients with severe scurvy is distinctive. Stasis cyanosis in the extremities, sallow dirty gray cadaveric skin color, somnolence, lethargy, and hypotension appear insidiously and are the prodromas of peripheral vascular collapse which may occur suddenly without further warning. Cheyne-Stokes type of respiration occurs particularly in patients with arteriosclerotic cerebrovascular disease and anemia. All of these vasomotor abnormalities disappear within from twenty-four to thirty-six hours after the oral or parenteral administration of adequate amounts of vitamin C. Although the exact mechanism responsible for these changes is unknown, it should be noted that a direct relationship has been reported to exist in guinea pigs and

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After a week on the vitamin C restricted diet, the patient developed respiratory irregularity and peripheral cyanosis. On the tenth day he became very dyspneic even after the slight exertion of sitting up in bed. When resting, the respirations were of the Cheyne-Stokes type. He was confused and somnolent. The blood pressure was 80/50. Administration of vitamin C, 500 mg. daily in divided doses intramuscularly, was begun. Thirty-six hours thereafter the respirations were regular except for an occasional apneic period. The blue-red color about the tooth snag had faded, and the swelling had decreased. The lead gray color of the skin and cyanosis of the nails had disappeared. Blood pressure was 108/60. Three days after vitamin C, respirations were entirely normal, and the patient was well orientated. Blood pressure was 115/65. In four days the icteric index was 5, and urine urobilinogen was within normal limits. The gum and skin lesions healed completely in twelve days. Blood counts were normal in three weeks.

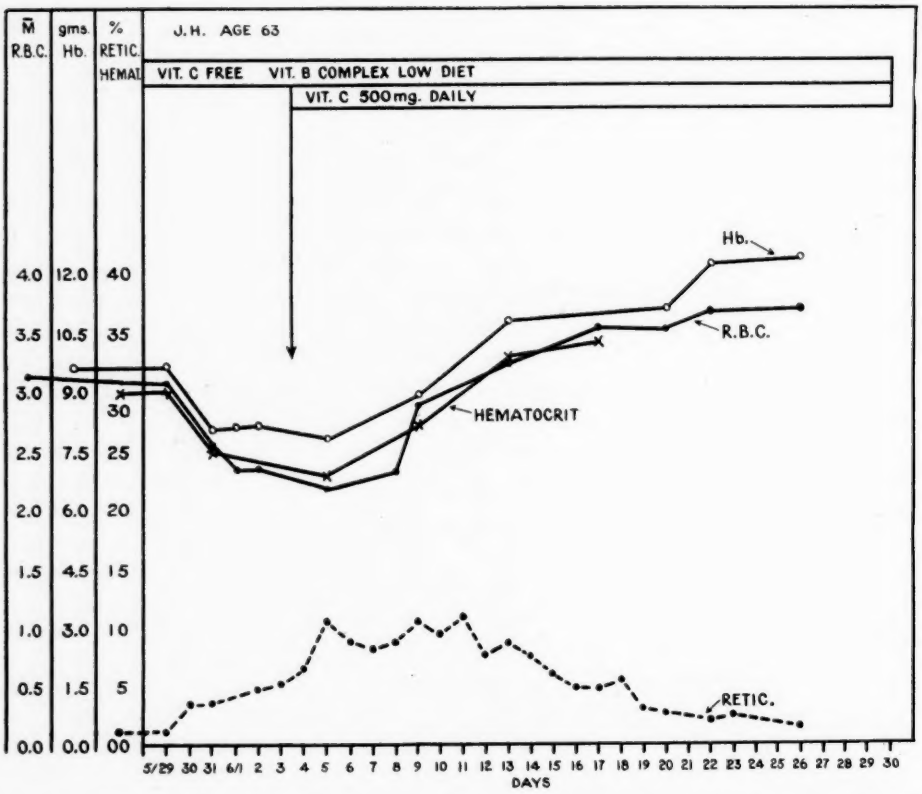

Fig. 6.—A patient with severe scurvy whose erythrocytes and hemoglobin declined and reticulocytes rose as his clinical course grew worse prior to vitamin C therapy. Thereafter, he made a rapid hematologic and clinical recovery.

J. H. (Case 15), a 63-year-old white man, was admitted to the Cincinnati General Hospital May 27, 1944, because of swelling of the legs and pain in the knees of six months' duration. For one year he had noticed easy bruising and for the previous six months numbness and tingling in the feet and legs. He ate oranges or grapefruit only when the cost was 15 cents a dozen. Breakfast consisted of coffee, canned milk, and doughnuts; dinner, soup, occasionally meat, potatoes, beans, bread, and beer; supper, a sandwich and beer. His wife had died in 1918, and he had been cooking for himself ever since.

The temperature was 101° F.; pulse, 116; respirations, 22; and blood pressure, 108/74. There was an 8 by 6 by 4 cm. deep-seated hematoma on the right forearm, and there were large ecchymotic areas as well as perifollicular hemorrhages over the thighs and lower legs. The ankles were moderately edematous. The sclerae were icteric and the mucous membranes pale and slightly cyanotic. The gums were swollen, blue-red, and spongy and bled easily. Neurologic examination showed only absent Achilles jerks and diffuse tenderness over the lower legs, especially in the regions of the ecchymoses. Later, distinct stocking type of hyperesthesia was found.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. No vitamin C was found in the plasma. X-ray of the chest showed moderate congestion at the lung bases. The tourniquet test was positive.

The patient was fed the vitamin C restricted diet during the entire hospital stay. After eight days the blood pressure had fallen to 90/50, and he was becoming confused. The skin was a sallow gray color. The erythrocyte count and hemoglobin level had fallen, and the reticulocyte count had risen to 5 per cent. Vitamin C was begun in dosage of 500 mg. a day orally, and the patient recovered over a period of three weeks. Four days after vitamin C was begun the blood pressure was 110/64. Icteric index was normal.

rats between the vitamin C stored in the adrenals and the synthesis of adrenocortical steroids.[12, 13]

In 1930 Mettier, Minot, and Townsend[9] concluded that scorbutic anemia responded specifically when orange juice was administered but did not respond to iron or purified liver extract. They showed that the erythrocytes were normocytic normochromic or moderately macrocytic hyperchromic, in contrast to previous teaching that the cells generally were hypochromic. They found the bone marrow moderately hyperplastic and normoblastic and noted an increase in cellularity due principally to an increase in normoblasts after vitamin C was administered.

Since 1930 numerous observers[14-22] have reported hematologic studies on one, two, or three persons with scurvy which had occurred naturally or had been induced experimentally.[23] The most comprehensive recent study involved fifty-three scorbutic patients in Edinburgh, Scotland.[24] Much of this work seems to indicate that scurvy and anemia do not necessarily coexist, that experimental vitamin C lack does not interfere with blood formation, and that patients with naturally occurring scurvy and anemia show erythrocyte and hemoglobin regeneration while on a vitamin C free but otherwise adequate diet. The bone marrow has been described as hyperplastic with normoblastic maturation arrest,[9] as hypoplastic,[21, 25] and as megaloblastic.[16] In short, the inference is that vitamin C is not essential for normal hematopoiesis and that hemorrhage, lack of iron, and some unknown vitamin B complex or other deficiency state account for scorbutic anemia.

The hematologic data gathered from our severely scorbutic patients while they were on a diet very low in vitamin C and low in the vitamins of the B complex corroborate many of the original observations of Mettier, Minot, and Townsend.[9] Nine of our critically deficient patients either did not improve clinically or hematologically or became more anemic and debilitated on this diet. Striking hematologic and clinical recovery occurred after vitamin C alone was added to the experimental regime, much the same effect previously reported for orange juice.

In addition, almost all of our patients had signs suggesting accelerated blood destruction, namely, reticulocytosis, slight or moderate jaundice, elevated urobilinogen output in urine and stool but no bile in the urine. All of these signs disappeared within from four to twelve days after vitamin C was administered. None of the patients had clinical evidence of hepatic dysfunction, and of twenty-nine liver function tests performed on eleven patients, only nine tests were definitely abnormal. Such hepatic dysfunction as was found by laboratory procedures was no more than might have occurred from chronic nutritional deficiency. Liver disease could scarcely have been responsible for all of the signs associated with the jaundice. These data suggest that hemolysis was partially responsible for the anemia and that this factor was rapidly eliminated by the administration of vitamin C. Since neither the degree of the anemia nor the degree of the jaundice could be correlated with the extent of visible ecchymoses, it seems possible that some of the hemolysis occurred in-

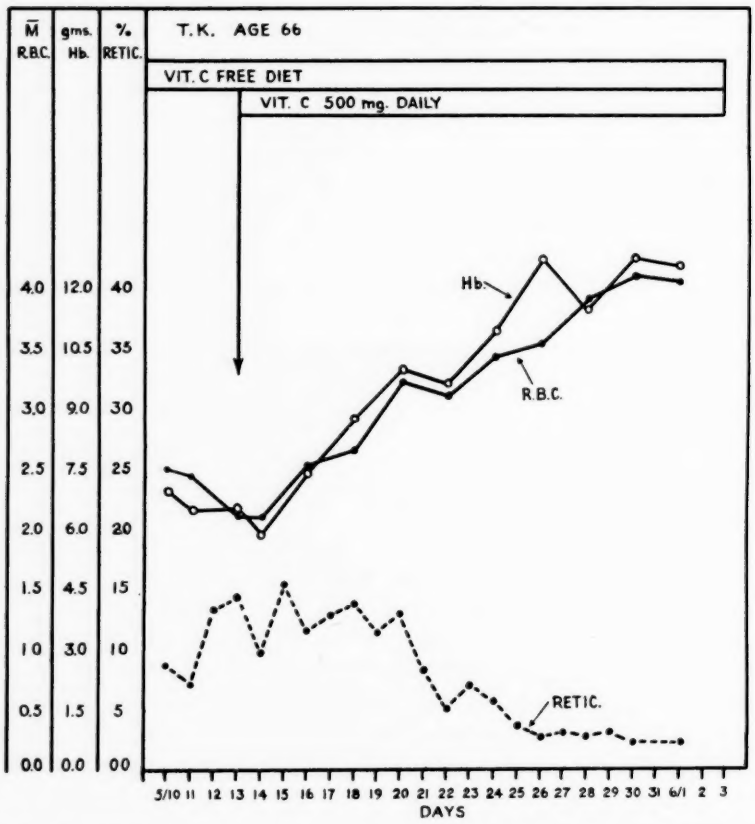

Fig. 7.—A patient with severe scurvy whose erythrocytes and hemoglobin declined and reticulocytes rose as his clinical condition grew worse prior to the administration of vitamin C. Thereafter, he made a rapid clinical and hematologic recovery.

T. K. (Case 19), a 66-year-old white man, was admitted to the Cincinnati General Hospital May 10, 1945, because of inability to walk, tenderness in the calves, and numbness in the feet. The appetite had been poor, and he had been eating nothing but vegetables, soup, cheese, bread, and beer for six months. Prior to that the chief articles of diet were beans, sauerkraut, and potatoes. Two years before admission he quit work because of weakness in the legs. He was living with his wife, but his daughter, who had been caring for the old folks, had left for the WACs one year before. He was unable to remember when he had last eaten citrus fruits or tomatoes.

The temperature was 100.6° F.; pulse, 90; respirations, 24; blood pressure, 190/100. The respirations were of Cheyne-Stokes type. The skin over the legs and thighs was covered with large, deep, dark purpuric spots with white firm centers. The gums were blue-red, swollen, and very tender. The sclerae were icteric. The skin was a dirty yellow-gray color, nail beds were cyanotic, and there were splinter hemorrhages under the nails. The chest was emphysematous. The patient was confused and disoriented. The arms and legs were somewhat spastic. All deep reflexes were hyperactive. There were bilateral positive Hoffmann reflexes and bilateral ankle clonus.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. The plasma vitamin C level was 0.0 mg. per cent; blood urea nitrogen 18 mg. per cent. Electrocardiogram showed myocardial dam-

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travascularly rather than solely in the ecchymoses, although this has not been proved.

The bone marrow was never as hyperplastic as one would expect in patients with pure hemolytic jaundice or the anemia of acute blood loss. In fact, the marrow sample obtained by sternal aspiration was definitely hypocellular in one patient who had distinctly elevated reticulocytes. If this specimen represented adequate sampling of all the marrow, then definite hypocellularity was present. White blood cell counts frequently were low. These observations suggest that there were significant etiologic factors other than hemolysis. All of these abnormalities disappeared when vitamin C alone was administered to patients whose previous course had been stationary or downhill. The conclusion seems inescapable that vitamin C reversed these abnormal processes and is essential for normal formation and maintenance of erythrocytes.

On the other hand, we too have found that patients with mild or moderate scurvy may have no anemia or may improve clinically and hematologically when they are put to bed and fed a vitamin C restricted diet. In fact, a normal blood picture has been found in twelve ambulatory patients with scurvy who have visited the Nutrition Clinic, Hillman Hospital, Birmingham, Ala.* Further observations from this clinic indicate that many nutritionally deficient persons may have repeatedly negative tests for vitamin C in the plasma for five years and yet develop neither clinical scurvy nor anemia.

The factors which cause the development of anemia in some persons with scurvy but not in others are not clearly understood. Certain considerations, however, help explain this variation in hematologic response. In the normal course of events deficiency diseases seldom occur as single entities. Deficient diets are seldom lacking in a single essential factor. The altered physiology induced by the deficiency of one essential nutrient may increase the need of the cells for the other essential nutrients and may exaggerate the pathologic response of the cells if a second or third deficiency occurs. As an example,

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age, S-T_1 and S-T_4 depressed; QRS, .10 second; P-R interval, .20 second. Cerebrospinal fluid was normal.

The patient was placed on the vitamin C restricted diet for the entire hospital stay. After four days, periodic breathing was more pronounced and he was much more confused, the nail beds more cyanotic, and the blood pressure 140/90. Erythrocyte and hemoglobin levels had fallen and reticulocytes had increased to 14 per cent. He told the examining physician that he would not live until the next morning. Vitamin C in doses of 100 mg. five times a day was given by mouth May 14, 1945. After twenty-four hours the peripheral cyanosis and muddy color of the skin had disappeared. The gums were less swollen and were paler. Sclerae appeared to be less icteric. The patient was less confused and was breathing normally.

In six days the gums had completely healed and the purpuric areas had become brown. All signs of jaundice had disappeared and the icteric index was 5. The patient made a rapid recovery from scurvy, but neurologic manifestations were unaltered.

*These studies were supported by grants from Chas. Pfizer & Co., Inc., New York, N. Y.

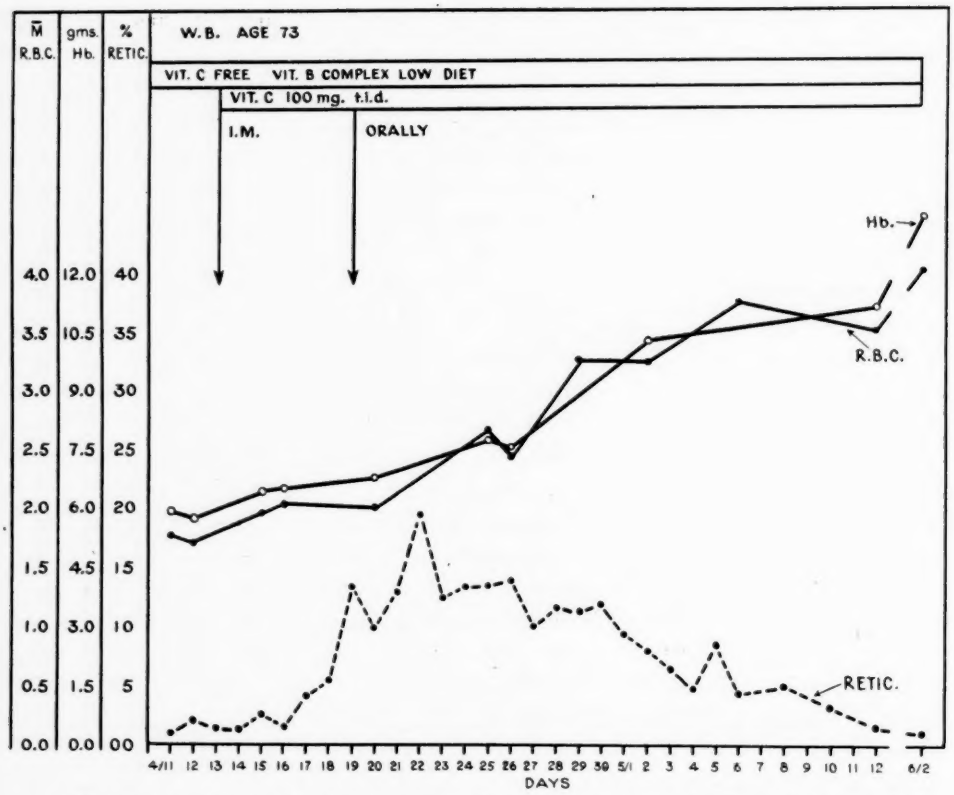

Fig. 8.—A patient with severe scurvy. Ten days after vitamin C was begun, reticulocytes had increased to a maximum of 19.2 per cent. Thereafter, the erythrocytes and hemoglobin increased rapidly. Such a reticulocyte response following the administration of vitamin C was observed in only one other patient.

W. B. (Case 11), a 73-year-old white man, entered the Cincinnati General Hospital April 4, 1944, because of pain and weakness in the knees. One year before he had noted black and blue marks about each knee which appeared without trauma. Recently these areas had become larger and the knee joints painful. He had never married and had always eaten in restaurants. He stated that he liked fruits and vegetables but could not afford to eat them.

His temperature was 99.8° F.; pulse, 98; respirations, 20; and blood pressure, 190/94. The inner aspects of the thighs and both knees were covered with confluent ecchymoses associated with phlebothrombosis in the popliteal regions. There were a few perifollicular hemorrhages over the tibiae and small subcutaneous ecchymoses on the right elbow, left forearm, right temple, and right upper eyelid. The mucous membranes were pale and the sclerae were jaundiced. Gums were normal, even around one tooth remnant. The liver was moderately enlarged but nontender. Signs of old left hemiplegia were found.

The data concerning peripheral blood, liver function and bile pigment studies, and bone marrow are summarized in Tables II, III, and IV. The vitamin C level in the plasma was 0.0 mg. per cent. Blood urea nitrogen was 26 mg. per cent. Gastric analysis showed 22° of free hydrochloric acid after histamine stimulation. A barium enema and gastrointestinal series showed only distended bowel.

The patient received the vitamin C restricted diet during the entire period of hospitalization, and administration of vitamin C, 100 mg. three times a day intramuscularly, was begun April 13, 1944. Ten days later the reticulocytes had mounted to 19.2 per cent, and twelve days after administration of vitamin C the icteric index and urine urobilinogen values were normal. The patient was discharged June 2, 1944, in good condition.

a patient with severe vitamin C depletion may have no anemia until additional strain is placed on the bone marrow by a deficiency of extrinsic factor, protein, iron, or other unknown factors which may be necessary for normal hematopoiesis. Yet, after anemia has developed, the deficiency of the latter factors may not be serious enough to prevent a remission when large amounts of vitamin C are administered. In many deficient persons, bed rest which reduces metabolic requirements for all essential nutrients may be sufficient therapy to produce a clinical and hematologic remission. Depending on the interplay of multiple factors, morphologic differences in blood and bone marrow and varied therapeutic responses may readily occur in patients with scurvy and other deficiency states. For these reasons, our observations on patients who were critically ill with scurvy, anemia, and other deficiency diseases of long standing cannot be compared satisfactorily with data on human subjects in whom a single deficiency state, scurvy, has been produced experimentally[23] without the occurrence of anemia. For all these reasons, the stage is set for the accumulation of data which appear to be contradictory. The difficulties of establishing the exact relationship of vitamin C to the anemia of scurvy are multiplied. Our data indicate that a deficiency of vitamin C is the major factor in the development of scorbutic anemia such as was found in these patients, although the modus operandi remain unsettled.

SUMMARY AND CONCLUSIONS

1. The prodromal period and the clinical features of severe scurvy are described in nineteen scorbutic patients admitted to the Cincinnati General Hospital over the past ten years. Perifollicular hemorrhages, ecchymoses, swollen painful joints, and swollen blue-red tender gums occurred frequently. Blood pressures tended to be low, and many of the patients were bordering on vascular collapse.

2. All but two of the nineteen patients had anemia.

3. Complete hematologic studies were carried out on eleven patients. The anemia was normochromic and normocytic or slightly macrocytic. Persistent reticulocytosis, moderate leucopenia, and thrombopenia occurred frequently. Moderate icterus of the hemolytic type was noted in most of the patients.

4. The bone marrow appeared moderately hypocellular in one patient and normally cellular or hypercellular in the others. Differential counts of nucleated marrow cells indicated a relative increase in normoblasts and, to a lesser extent, in the younger members of the red cell series.

5. Two patients with mild or moderately severe scurvy improved clinically and hematologically after bed rest.

6. In nine severely scorbutic patients, despite persistent reticulocytosis, erythrocyte counts and hemoglobin levels were unchanged or fell while the patients were in bed on a vitamin C restricted diet.

7. Hematologic and clinical improvement occurred in each of these patients after crystalline vitamin C was administered.

8. The concept of the multiple deficiency state, with many factors besides vitamin C deficiency adversely affecting the bone marrow, can explain the many conflicting reports on scorbutic anemia which have appeared since 1930.

9. In these eleven consecutive patients the anemia of scurvy occurred primarily because of vitamin C deficiency.

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THE EFFECT OF FOLIC ACID (LACTOBACILLUS CASEI FACTOR) IN NUTRITIONAL HEMATOPENIA OF MONKEYS

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PREVIOUS reports from this and other laboratories[1-8] have described the clinical and hematologic aspects of the nutritional deficiency which develops in monkeys when they are maintained on diets deficient in certain constituents of the vitamin B complex. Day and associates[1-5, 9] have shown that vitamin B deficient diets supplemented with niacin, thiamine, and riboflavin failed to prevent nutritional cytopenia, whereas liver extract or brewers' yeast did prevent these changes. The essential but unidentified component in these two substances was designated vitamin M. Subsequently, in 1942, it was reported from this laboratory[6-8] that the addition of calculated supplements of niacin amide, thiamine, riboflavin, calcium pantothenate, pyridoxine, inositol, paraminobenzoic acid, choline, pimelic acid, and glutamine (Diet 2) failed to prevent the progressive development of the clinical signs of deficiency or the hematopenia, although on this diet leucopenia appeared more consistently in the monkeys in our studies and was usually more pronounced than anemia. Thus, it became evident that the essential component present in liver and yeast was not identical with, or completely included in, these additional individual or collective constituents of the vitamin B complex. Our observations at that time suggested that a folic acid concentrate of yeast was effective in "re-establishing a normal white cell equilibrium" and that this folic acid "closely simulated the effect of parenteral liver extract."[6, 8] It has since been reported that *Lactobacillus casei* factor has been used effectively in completely compensating for the hypothetic vitamin M deficiency in the monkey.[9] The purpose of this communication is to report in more detail our studies of the effects of folic acid concentrate of yeast and of crystalline *L. casei* factor from liver in nutritional hematopenia in the monkey.

Following a preliminary period of observation, five monkeys were placed on a synthetic diet and were studied for nutritional changes in the same manner, using the same techniques of feeding as have been previously described.[6] Two of the animals (Monkeys 53 and 136) received the vitamin B free basic diet, supplemented with all of the separate constituents of the vitamin B complex previously listed (Diet 2), while three animals (Monkeys 61, 1C, and 96) were given the basic diet alone, supplements being added only after signs of nutritional deficiency appeared.

Three of the five animals (Monkeys 53, 61, and 136) were treated intramuscularly with a folic acid concentrate* from yeast[10] containing approximately 57 μg of *L. casei* factor per cubic centimeter. The other two (Monkeys 96 and

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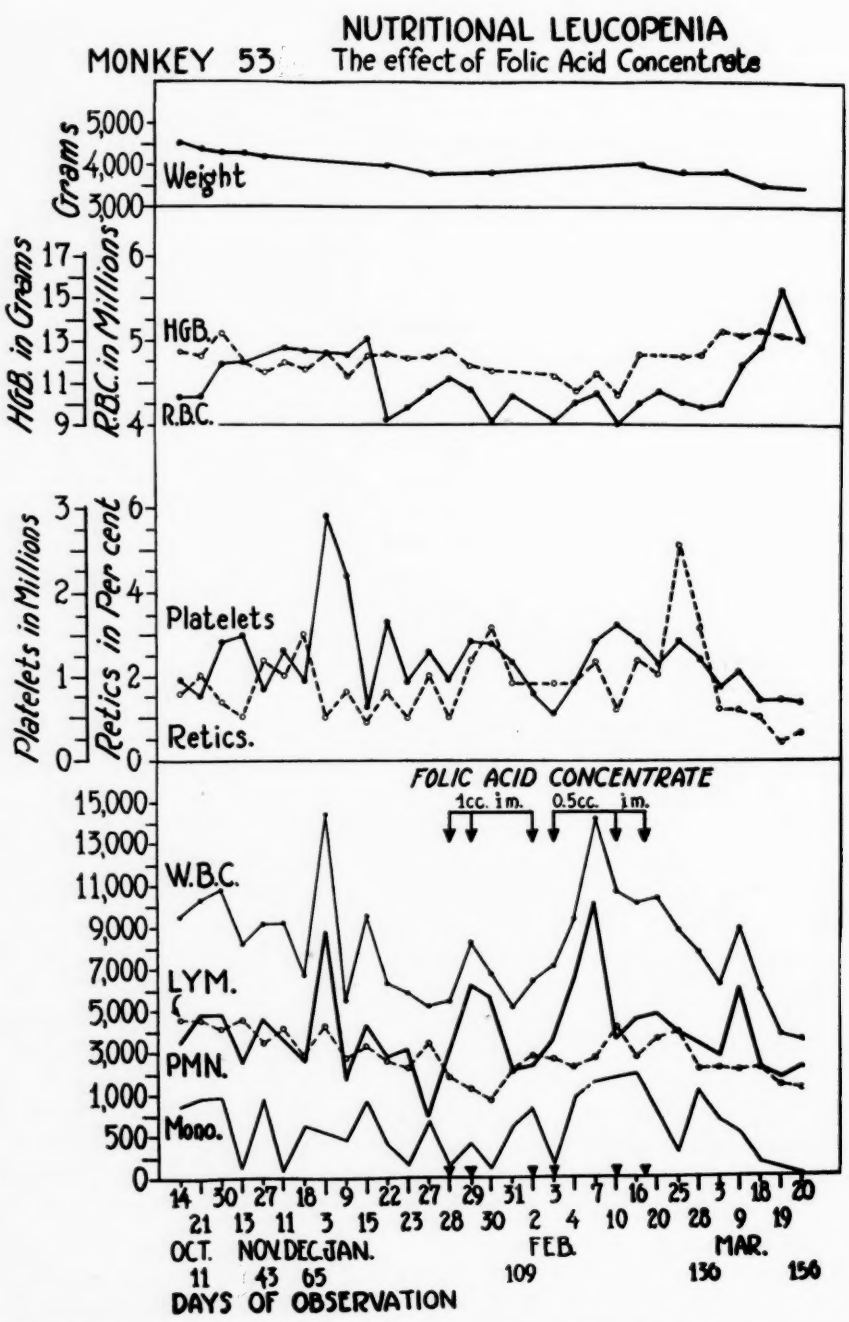

Fig. 1.—This animal on Diet 2 showed a progressive granulopenia and lymphopenia with only a minimal degree of anemia. The initial granulocyte response occurred twenty-four hours and the maximum eleven days after starting folic acid concentrate.

1C) were given a highly purified crystalline preparation of *L. casei* factor[11] intravenously.*

Monkey 53 (Fig. 1), while on Diet 2, showed gradual weight loss and later some diarrhea during the first seventy diet days, but the general appearance and vitality of the animal remained fairly good. Despite the wide fluctuations in granulocytes, the total W.B.C., granulocytes, and lymphocytes fell progressively, until by the eighty-eighth day of diet the white cells had fallen from a total of 10,350 with 4,864 neutrophils and 4,243 lymphocytes to 5,150 W.B.C. with only 669 granulocytes and 3,502 lymphocytes.

Intramuscular injections of folic acid concentrate totaling 4.5 c.c. were given in six divided doses over a period of twenty days (Fig. 1). There was a prompt increase in granulocytes which reached a peak of 10,080 (higher than normal base line levels) eleven days after the first injection. Following discontinuance of the folic acid, there was a progressive decline in the total white cells which, however, remained within normal limits for twenty-nine days after the last injection.

There was a minimal degree of anemia at the time the folic acid concentrate was given. The red cells did not fall below 4.0 million, as compared with base line levels of from 4.3 to 5.0 million, while hemoglobin values fluctuated between 11.5 and 12.5 Gm., as compared with base line values of from 13.2 to 14.0 Gm. There was a slight increase in reticulocytes to 3 per cent within three days and a secondary rise to 5.4 per cent twenty-eight days after beginning the folic acid injections. There followed an increase in total red cells to 5,700,000. After this brief period of hematologic and clinical improvement, there was progressive decline and ultimate death of the animal (Monkey 53). In this monkey the granulocytes and lymphocytes were the most sensitive index to the dietary deficiency in an essential nutritional and hematopoietic component, and accordingly they reflected a more immediate, though transitory, response to folic acid supplement than the erythroid elements or the thrombocytes.

Monkey 136 was maintained on Diet 2 for 135 days prior to the experimental studies charted in Fig. 2. During that initial period the animal gradually developed clinical signs of nutritional deficiency, including anemia, leucopenia, and thrombocytopenia. The weight declined from 3,060 to 2,480 grams. The total white cell count fell from a base line average of from 10,200 to 5,000 and the granulocytes from 6,400 to 3,600, and the lymphocytes were reduced from 2,000 to 720 per cubic millimeter. Similarly, the red cells gradually dropped from 4.5 to 5.0 million to a low point of 2.9 million and the hemoglobin from 12.5 to 14.0 Gm. to 7.8 Gm. The platelets fell from 1,000,000 to 200,000 per cubic millimeter. After the signs of nutritional deficiency and panhematopenia were thus well advanced, crude liver extract was administered intramuscularly daily, for eight days, and the animal was returned to a normal diet for thirty days. A prompt reticulocyte crisis followed with a transitory overcompensatory granulocytosis and thrombocyte recovery. Coincident with this hematologic response, there was marked clinical improvement. This monkey,

*We are indebted to Dr. E. L. R. Stokstadt for this material.

however, did not regain the weight lost during the first period on Diet 2, and there was a persistent diarrhea. When the animal was again placed on Diet 2, granulocytes, lymphocytes, and erythrocytes fell again promptly. On the fifty-fourth diet day folic acid concentrate injections were begun; they resulted in an immediate significant granulocytosis and a later rise in red cells, hemoglobin,

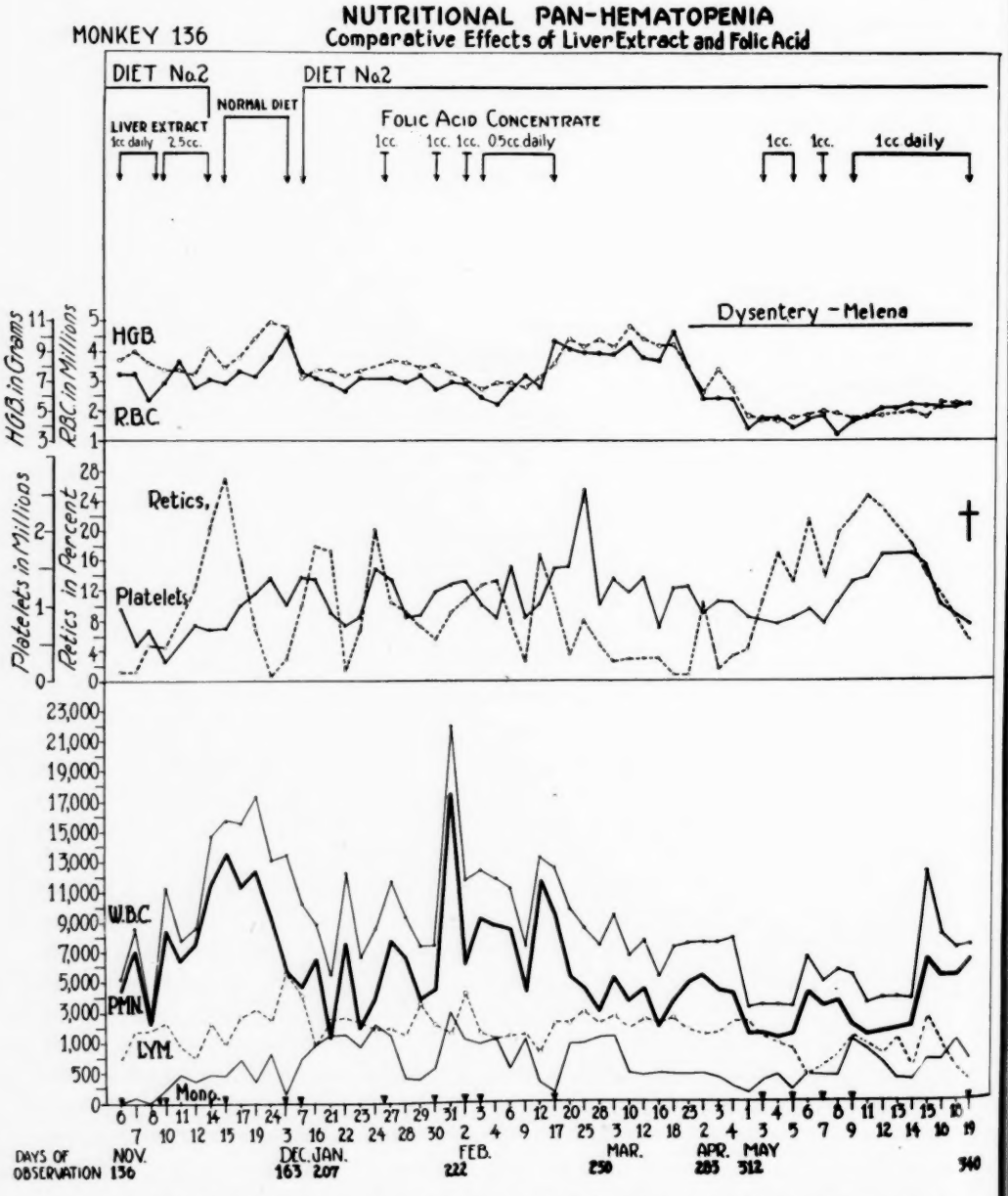

Fig. 2.—The response to the first series of folic acid concentrate injections is strikingly similar to that of liver extract, with an increase in all of the white cell elements, red cells, hemoglobin, and the blood platelets.

and platelets comparable to that noted following the earlier liver extract injections. The first series of daily folic acid concentrate injections was stopped on the seventy-fourth day of diet and was followed by a decline in granulocytes. The clinical state of the animal deteriorated rapidly, and the decline was accelerated by a spontaneous *Shigella dysenteriae* infection. The loss of blood in the feces at this time precipitated a profound anemia. The bone marrow study (April 3, 1942) revealed an absolute hypoplasia of both the erythroid and myeloid elements with a slight left shift in the erythroid elements. In spite of the fact that the animal was in a profound state of debility, another series of folic acid concentrate injections was begun and resulted in a reticulocyte crisis with a peak of 24 per cent and an abortive granulocytosis and thrombocytosis. These reactions were quantitatively less than the earlier marrow responses to liver and folic acid, respectively, and inadequate to initiate recovery from the established intestinal infection. Terminally, a study of the femoral bone marrow revealed a more advanced degree of erythroid and myeloid hypoplasia than had been observed seven weeks earlier.

Monkey 61 (Fig. 3) was maintained on the basic diet, supplemented for the first few days of the experimental period by oral yeast. On this regime diarrhea and leucopenia developed and led to a very rapid decline in the clinical state of the animal. An optimum diet was reinstituted with prompt clinical improvement and a moderate reticulocyte increase and hemoglobin rise but with the white cell levels increased only slightly. The basic diet was then resumed without vitamin B supplements, and by the end of four months a relatively profound anemia had developed.

During this period the animal showed several distinct episodes of clinical decline associated with a further progressive leucopenia, with transitory remissions in the leucopenic phases paralleled by temporary clinical improvement (Fig. 3). On Feb. 24, 1942, the total white blood cells fell to 6,350, as contrasted with base line levels of from 11,000 to 16,800, and the neutrophils fell from a base line of from 6,500 to 1,524 per cubic millimeter. Again on May 19, 1942, the leucocytes fell to 8,500 and the granulocytes to 1,400. By the one hundred twenty-eighth day on this diet there was marked anemia and leucopenia and clinical debility. At this point folic acid concentrate was started. The animal received 2.0 c.c. intramuscularly daily, for eight successive days; then after a lapse of one week the injections were continued, 2.0 c.c. every second day for a period of fifty-four days. After the administration of this material, there was a sharp immediate response in granulocytes, followed shortly by an increase in reticulocytes and platelets. The granulocytes reached a peak of 16,000 with the total white cells 19,250 in an overcompensatory rebound on the eighth day of therapy and then returned to base line levels (from 4,000 to 5,000), coincident with an increase in lymphocytes up to 10,000 per cubic millimeter. A marked increase in thrombocytes to 3,700,000 per cubic millimeter coincided with the maximum reticulocyte response of 25.6 per cent which was observed after nine days. There followed an increase in circulating red cells to 5.5 million from 3.2 million and in hemoglobin to 14.4 Gm. from 8.9 Gm. after folic acid supplements. Following this period of hyperresponse, the animal was returned to an

optimum diet supplemented by parenteral liver extract. The red cells and hemoglobin resumed their normal equilibrium. There was gradual clinical improvement and weight gain over a period of six weeks after beginning folic acid concentrate, but at no time did the animal (Monkey 61), completely lose the hyperkeratosis and scaling of the skin which had appeared during the period of deficiency nor completely regain its normal weight and appearance of well-being. The anemia in this monkey on a limited basic diet was more pronounced

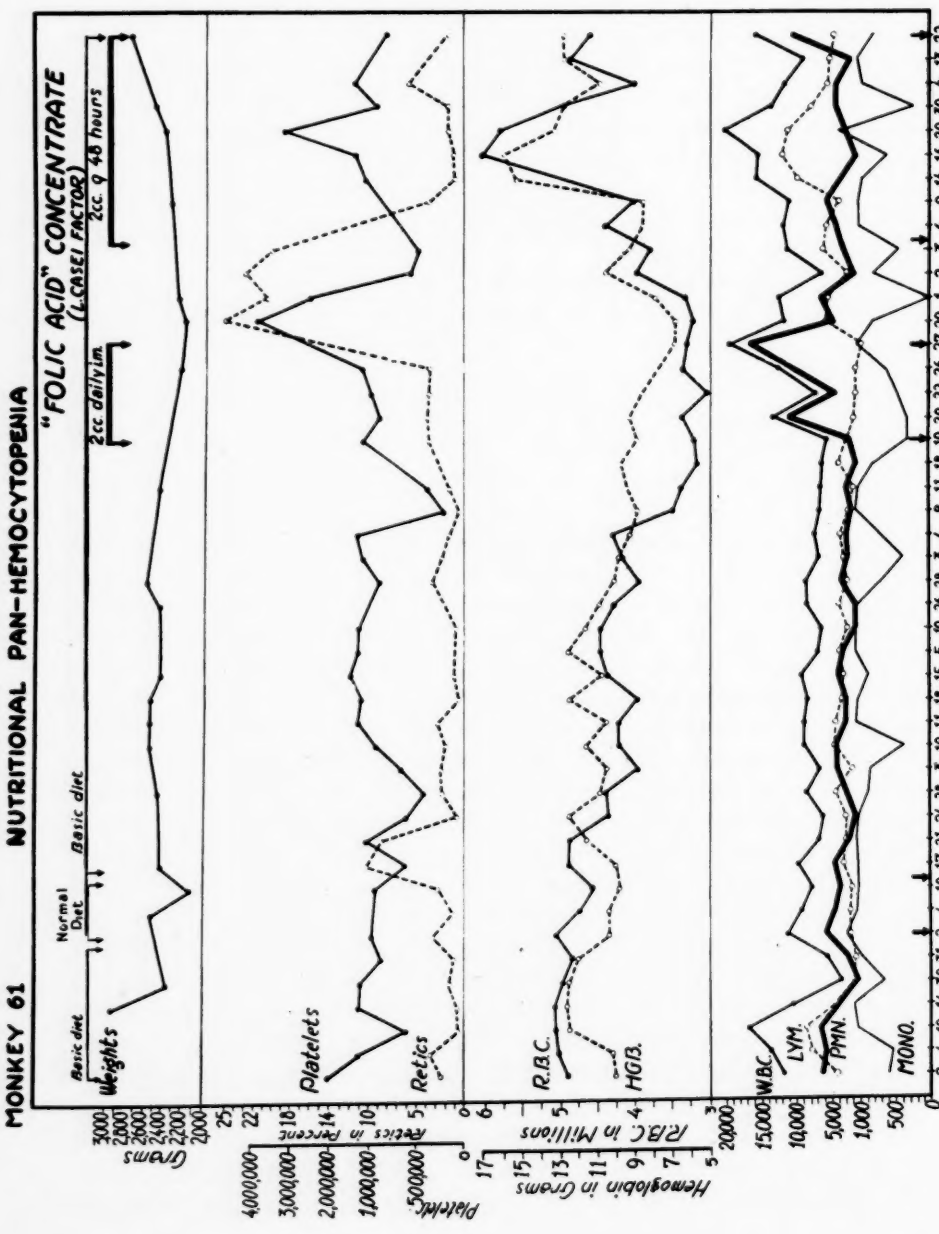

Fig. 3.—The first response to folic acid concentrate was an increase in neutrophiles within twenty-four hours, which was soon followed by crises in the reticulocytes and platelets. The increase in lymphocytes, red cells, and hemoglobin paralleled the weight gain and preceded the clinical improvement.

than that seen in most of those animals maintained on the basic diet plus supplemental B fractions, and the response of the erythroid elements to folic acid was correspondingly more dramatic.

COMPARISON OF THE EFFECTS OF ADENYLIC AND FOLIC ACID

The role of adenine and guanine nucleotides in myelopoietic stimulation has been experimentally demonstrated[12, 13] and the principle applied therapeutically[14-16] in recent years. Because of the apparent specific effect of *L. casei* factor on myelopoiesis in nutritional leucopenia, it seemed desirable to compare the effects of folic acid and adenylic acid under the conditions of this experiment.

Monkeys 96 and 1C were placed on basic diet without the vitamin B supplements in order to produce the deficiency syndrome rapidly. Both animals showed a similar response throughout the parallel periods of observation (Figs. 4 and 5). During a period of sixty-two days on this restricted diet the total white cells in Monkey 96 fell from an initial 18,000 to 21,000 base line to 6,900; the granulocytes, from 16,000 to 2,400; and the lymphocytes, from 3,900 to 1,100. A significant anemia also developed, the red cells falling from 5.5 to 6.0 to 2.88 million per cubic millimeter and the hemoglobin from 12.7 to 7.1 Gm. per 100 c.c.

At this time daily intravenous injections of adenylic acid (0.2 mg.) were started. During this part of the investigation complete blood studies were made four times daily. It was observed that for a few hours after each daily intravenous injection there was a temporary marked leucocytosis. The high granulocyte peaks recorded were obtained during these early postinjection periods. However, the lymphocytes remained abnormally low, and the granulocytes returned to leucopenic levels within from eight to twelve hours after each injection. The red cells, hemoglobin, reticulocytes, and platelets were unaffected.

Monkey 96 then was given daily intravenous injections of *L. casei* factor (from 0.1 to 0.2 mg., see Fig. 4). In response to this substance there was a progressive upward trend in both granulocytes and lymphocytes, with total white cells varying from 9,000 to 16,000 and granulocytes from 6,000 to 10,000, and lymphocytes remaining fairly stable at from 2500 to 3000 per cubic millimeter. There was an appreciable reticulocyte response (6.8 per cent after 0.7 mg. of *L. casei* factor had been administered over a period of twenty-two days, and a secondary reticulocyte peak occurred after further continued daily administration. A slow, but progressive, rise in red cells (from 4.5 to 5.5 million) and hemoglobin (from 12.6 to 13.0 Gm.) took place during this seventy-day period of *L. casei* factor administration. The clinical state of the animal improved coincident with the hematopoietic recovery, and this improvement continued and was maintained after the addition of the other vitamin B supplements. However, the animal was not entirely normal. There was marked irritability and a tendency to jerky spasmodic movements which did not disappear until this monkey had been on a normal diet for a number of weeks after the end of the experiment. Monkey 1C (Fig. 5) showed a somewhat more profound nutritional leucopenia and a less marked temporary leucocytosis in response to adenylic acid administration. However, the cumulative and sus-

Fig. 5.—The first response to folic acid concentrate was an increase in neutrophiles within twenty-four hours, which was soon followed by crises in the reticulocytes and platelets. The increase in lymphocytes, red cells, and hemoglobin paralleled the weight gain and preceded the clinical improvement.

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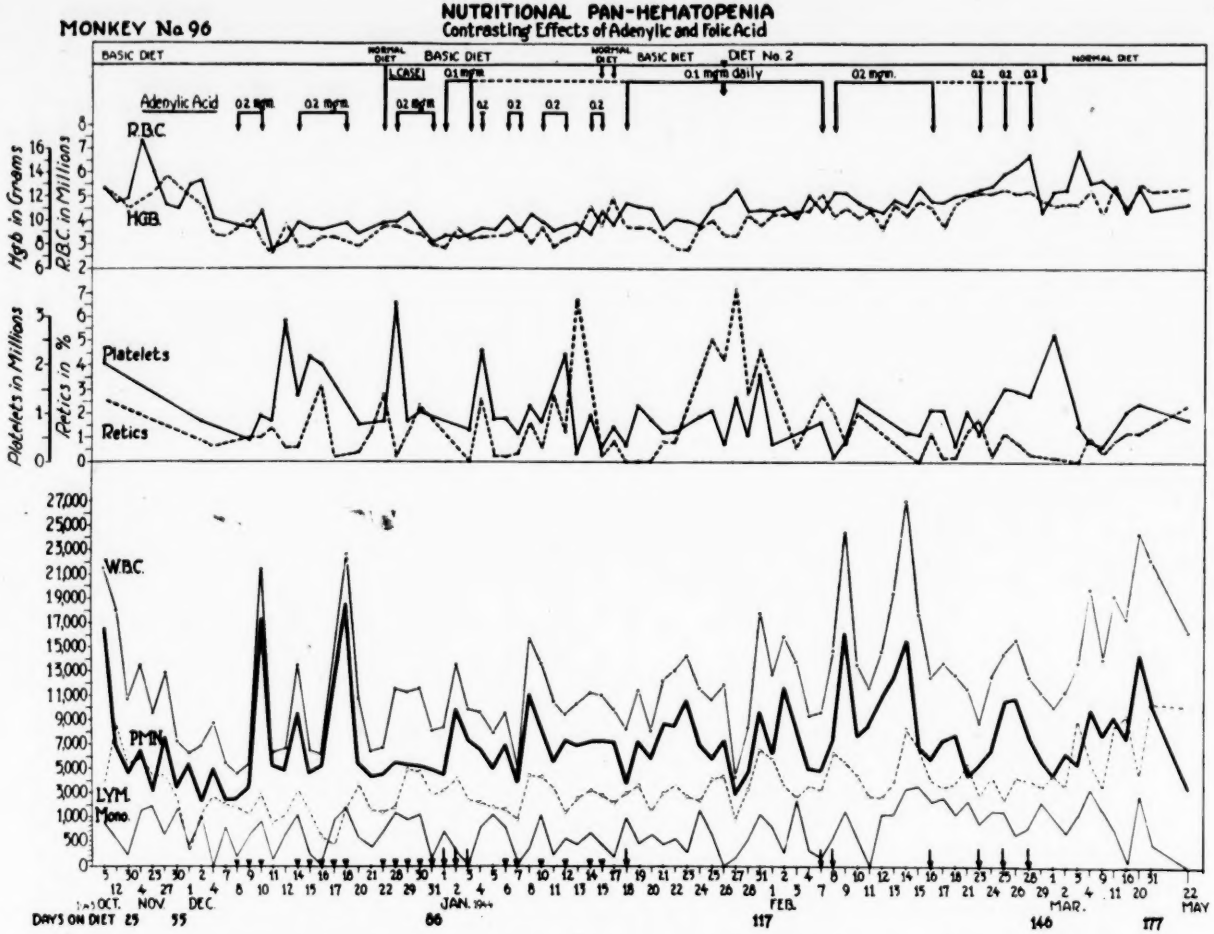
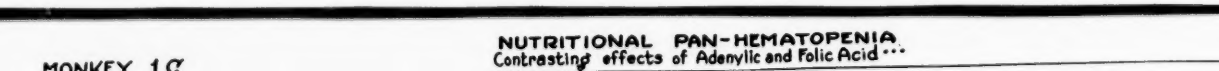


Fig. 4.—The response to each adenylic acid injection was a sharp transitory elevation of the granulocytes followed by a rapid return to leucopenic levels with no appreciable influence on the red cells. The response to *L. casei* factor was a progressive increase in neutrophiles and lymphocytes with a low reticulocyte peak followed by recovery of the red cells and hemoglobin. The subsequent addition of the other B complex supplements (Diet 2) seemed to augment the initial *L. casei* effect, both clinically and hematologically.



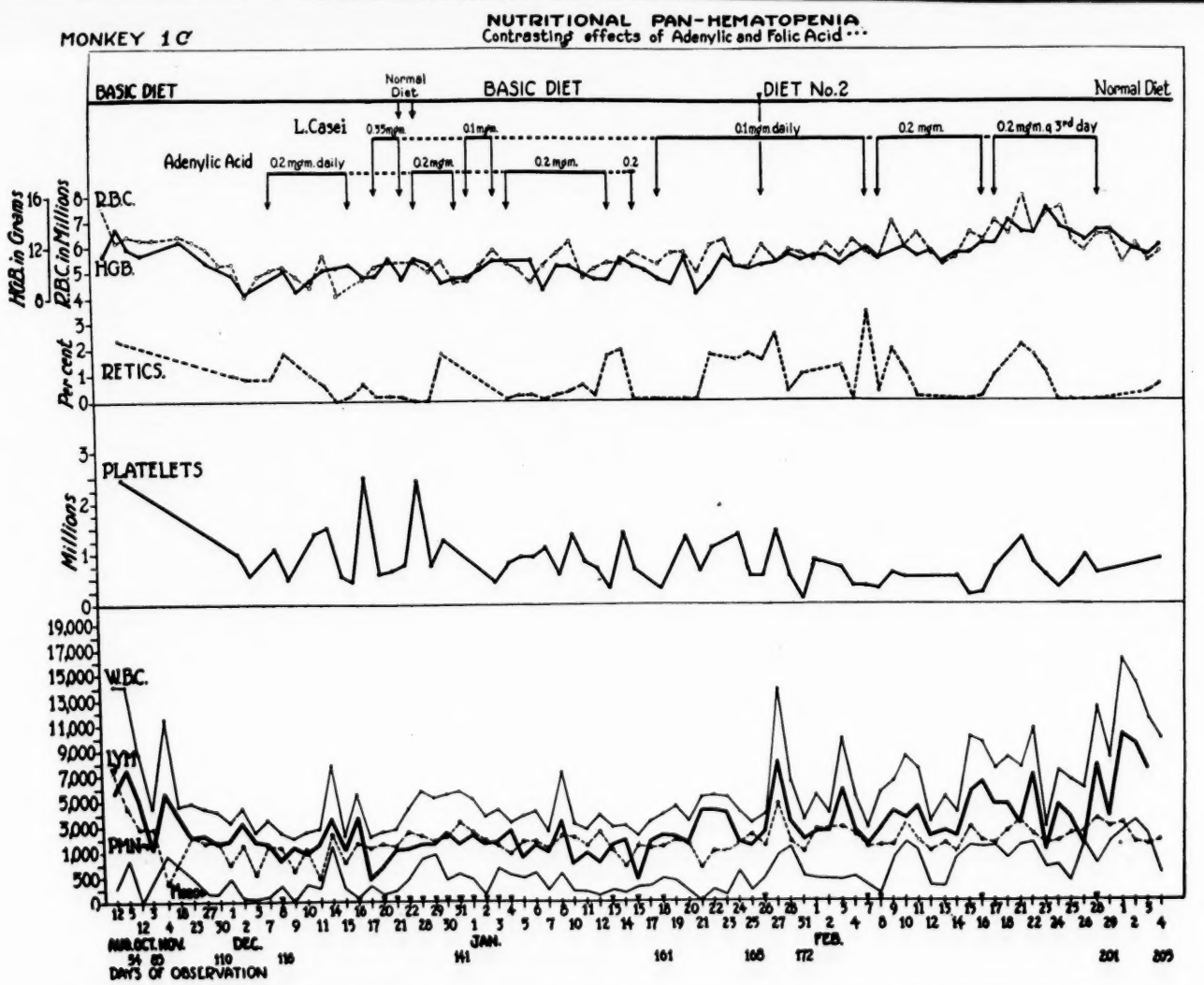


Fig. 5.—The nutritional neutropenia and lymphopenia were more profound in this animal than in Monkey 96 (Fig. 4). Therefore, the progressive recovery of the white cells, including the monocytes, and the steady rise in red cells was correspondingly more apparent and dramatic following the substitution of *L. casei* factor for adenylic acid.

tained erythroid and myeloid recovery following *L. casei* factor, as contrasted with adenylic acid, was as striking and definite as that in Monkey 96 just described.

In control studies three normal monkeys, each on optimum diet, were given daily intravenous injections of 0.2 mg. of adenylic acid over a period of several weeks. A marked transitory leucocytosis was observed for a few hours after each injection with subsequent return of white cell levels to normal within twelve hours.

Two other normal monkeys in an optimum nutritional state received repeated intravenous injections of 0.2 mg. of synthetic *L. casei* factor with no significant alteration in any of the peripheral blood cell levels.

DISCUSSION

All of the five animals on the experimental diets described herein developed clinical and hematologic evidences of nutritional deficiency. In these animals, as in many others which we have observed on similar diets, the neutropenia developed first. The lymphopenia which appeared independently, later, was less subject to exacerbations and remissions than the neutropenia and more accurately reflected the degree of nutritional and physical deficiency of the animal. The rapidity of development and the degree of the panhematopenic tendency, like the clinical signs, varied with the individual animal. Thus, one animal (Monkey 53) showed only the leucopenia; the other four monkeys showed anemia plus leucopenia; and two of these (Monkeys 61, and 136) developed a significant thrombocytopenia. Individual variation was also observed by Langston and co-workers who reported that "a few animals died before the anemia became marked" and that "the degree of anemia varied somewhat with the animal."[4]

The contrasting effects of adenylic acid, causing a transient leucocytosis, and of *L. casei* factor, which stimulated a gradual sustained increase in both myeloid and erythroid elements, are clearly demonstrated.

It is possible that the folic acid concentrate which was used in three of the animals may have contained minimal components of yeast other than *L. casei* factor. Whatever other substances may have been present, however, were utilized precisely the same as is *L. casei* factor, as indicated by the similar nature and identical character of the hematologic responses.

The report of Day and associates[9] indicates that the addition of purified *L. casei* factor to similarly supplemented basic diets constitutes an adequate diet for the monkey. While this substance in the quantities administered to the monkeys in our studies was not entirely effective in returning the animals to a completely normal nutritional state within the period of these observations, there was restoration of the bone marrow to normal function with the re-establishment of a normal equilibrium of the cells in the peripheral blood, associated with marked clinical improvement.

It is likely that the anorexia shown by monkeys in the well-defined vitamin M deficiency state results in decreased diet consumption and consequently a rela-

tive protein deficiency which may play some part in the clinical and hematologic pictures. Similarly, it is possible that a dietary deficiency in *L. casei* factor or substances utilizable as *L. casei* factor may precipitate a relative insufficiency in other components of the vitamin B complex, despite the addition of these to the basic diet. Whatever role these factors may play, it seems clear that the *L. casei* factor is an essential molecule for normal hematopoiesis and optimum nutrition in the monkey. Furthermore, it seems that the potential activity of *L.casei* factor comprises not only an essential antianemia element but also a very potent myelopoietic and thrombocytopoietic stimulus for optimum bone marrow function. The critical deficiency threshold in any given animal at any given time may differ for red blood cells, granulocytes, lymphocytes, and blood platelets. The hematologic and clinical manifestations which result from a diet lacking the essential hematopoietic elements reflect the degree of inadequacy of the vital raw materials and/or catalysts necessary for erythroid, myeloid, lymphoid, and megakaryocytic functions, respectively.

Other recent studies in monkeys[17] and clinical studies in human nutritional leucopenia[18] and in E. M. F. deficiency anemias[19-22] using synthetic *L. casei* factor[23] lend support to this concept of *L. casei* factor as an essential panhematopoietic substance.

SUMMARY

1. Five monkeys (*Macaca mulatta*) on vitamin B deficient basic diets supplemented in four cases by all of the known B vitamin fractions except *L. casei* factor developed clinical and hematologic evidences of nutritional deficiency.

2. Three of these animals treated with a yeast autolysate containing *L. casei* factor showed dramatic clinical remissions and the re-establishment of a normal hematopoietic equilibrium.

3. Two of these animals treated with purified crystalline *L. casei* factor from liver showed unequivocal clinical and panhematopoietic remissions after failure of adenylic acid to evoke more than a very transitory, noncumulative, or sustained leucocytosis.

4. These studies indicate that a monkey under the conditions of these observations may develop a deficiency in one or all of the bone marrow elements, any one or all of which may respond promptly and completely to *L. casei* factor activity.

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FURTHER OBSERVATIONS ON THE ANTIANEMIC PROPERTIES OF 5-METHYL URACIL

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FOR many years the subject of macrocytic hyperchromic anemia has been under intensive investigation in many laboratories and clinics. The relationship of nutrition to the formation of blood cells has been one of the many divergent lines of these investigations. Since 1940 we have been interested in the function of thymine* in this relationship, since it is a normal constituent of the body cell. We observed that the administration of 1 Gm. or less of 5-methyl uracil gave no hematologic response when given to patients with nutritional macrocytic anemia, the anemia of sprue, and pernicious anemia in relapse.[1] At a higher dose level a hematologic response was observed in patients with pernicious anemia in relapse.[2] The present report is concerned with the effectiveness of synthetic 5-methyl uracil as a hemopoietic substance.

The criteria used in the selection of the patients were as follows:

1. An erythrocyte level of 2.5 millon or less
2. A color index of 1.0 or more
3. A mean corpuscular volume of 100.0 cubic microns or greater
4. A mean corpuscular hemoglobin of 32 micromicrograms or more
5. A mean corpuscular hemoglobin concentration of 34 per cent or more
6. A histamine refractory achlorhydria and achylia
7. A normal oral glucose tolerance curve
8. The absence of acute infections

Using these criteria, we selected six patients for study. A complete dietary and medical history was obtained, and a physical examination was performed. Gastrointestinal x-rays, urinalyses, and stool examinations were essentially normal in all instances, and an icteric index obtained prior to treatment was slightly elevated in each case. Sternal bone marrow studies performed on each patient before treatment was instituted revealed the megaloblastic arrest characteristic of E. M. F.† deficiency anemia.

Each patient selected for study was hospitalized, and base line laboratory studies were done. The diet was rigidly controlled and contained no meat, meat products, fish, poultry, or raw vegetables. Blood studies were performed

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*Because of the confusion arising from the similarity in pronunciation between thymine (5-methyl uracil) and thiamine (vitamin B_1), we suggest that the name 5-methyl uracil be employed when referring to the former compound, and we shall use this term in the present publication.

†Erythrocyte maturation factor.

TABLE I. ORAL DOSAGE OF SYNTHETIC 5-METHYL URACIL ADMINISTERED TO SIX PATIENTS

CASE	COURSE OF TREATMENT	DAILY DOSE (GM.)	NUMBER OF DAYS	TOTAL DOSE (GM.)	REMARKS
1	1	1.0	7	7.0	No therapy given for period of 45 days between Courses 2 and 3
	2	6.0	12	72.0	
	3	10.0	11	110.0	
2	1	10.2	11	112.2	No therapy given for period of 17 days between Courses 1 and 2
	2	10.0	13	130.0	
3	1	4.5	13	58.5	No therapy given for period of 5 days between Courses 2 and 3
	2	10.2	5	51.0	
	3	10.0	11	110.0	
4	1	6.0	14	84.0	
5	1	12.0	8	96.0	
6	1	6.0	12	72.0	

daily as previously described.[3] Bone marrow studies on each patient were performed prior to treatment, on the second day after the peak reticulocytosis had occurred during treatment, and again after the reticulocyte concentration had subsided to less than 3 per cent in the peripheral blood. Immediately prior to administration 5-methyl uracil was weighed on an analytic balance and suspended in one-half glass of water. The doses administered varied in each case, as shown in Table I.

TABLE II. EFFECT OF ORAL ADMINISTRATION OF SYNTHETIC 5-METHYL URACIL ON PERIPHERAL BLOOD IN PERNICIOUS ANEMIA IN RELAPSE

CASE	COURSE OF TREAT-MENT	R.B.C. (MILLION)			HB. (GM.)			RETIC. (%)			TOTAL DOSAGE (GM.)
		INITIAL	AFTER 7 DAYS	FINAL DAY	INITIAL	AFTER 7 DAYS	FINAL DAY	FIRST DAY OF RISE	DAY OF PEAK	AT PEAK	
1	1	1.99	1.73	2.01 (8)	7.5	7.0	6.3 (8)	–	–	–	7.0
	2	2.01	2.19	2.88 (22)	6.3	8.1	10.4 (22)	6	11	14.2	72.0
	3	2.37	2.48	2.98 (11)	8.0	9.0	10.2 (11)	4	10	13.2	110.0
2	1	1.58	2.02	2.51 (17)	6.2	8.4	9.8 (17)	4	6	19.0	112.2
	2	2.25	2.33	2.74 (13)	9.8	9.0	10.4 (13)	5	8	14.2	130.0
3	1	1.68	1.92	2.01 (13)	7.5	8.6	9.0 (13)	6	11	9.0	58.5
	2	1.93	2.55	2.55 (7)	9.4	10.9	10.9 (7)	5	6	8.8	51.0
	3	2.38	2.28	2.70 (13)	10.4	10.3	10.7 (13)	6	9	5.4	110.0
4	1	1.86	2.02	2.34 (14)	5.8	6.0	7.5 (14)	4	11	16.0	84.0
5	1	1.34	1.61	1.60 (8)	5.4	6.6	6.8 (8)	3	7	31.2	96.0
6	1	2.22	2.55	2.79 (12)	10.7	11.3	12.0 (12)	2(?)	5	14.4	72.0

The hematologic effect of 5-methyl uracil was observed in six patients with pernicious anemia in relapse, as shown in Table II. Coincident with the reticulocyte response, there was in each case a definite upsurge in well-being, an improvement in appetite, and an increase in the patient's interest in his surroundings. The subjective and hematologic response to the oral administration of 5-methyl uracil was in every way similar to that following the administration of folic acid to patients with pernicious anemia in relapse.

Case 1. Reduplication of the Hematologic Response in the Same Patient to Large Doses of 5-Methyl Uracil.—This patient was given 500 mg. of 5-methyl uracil b.i.d. orally for a period of seven days without hematologic response.

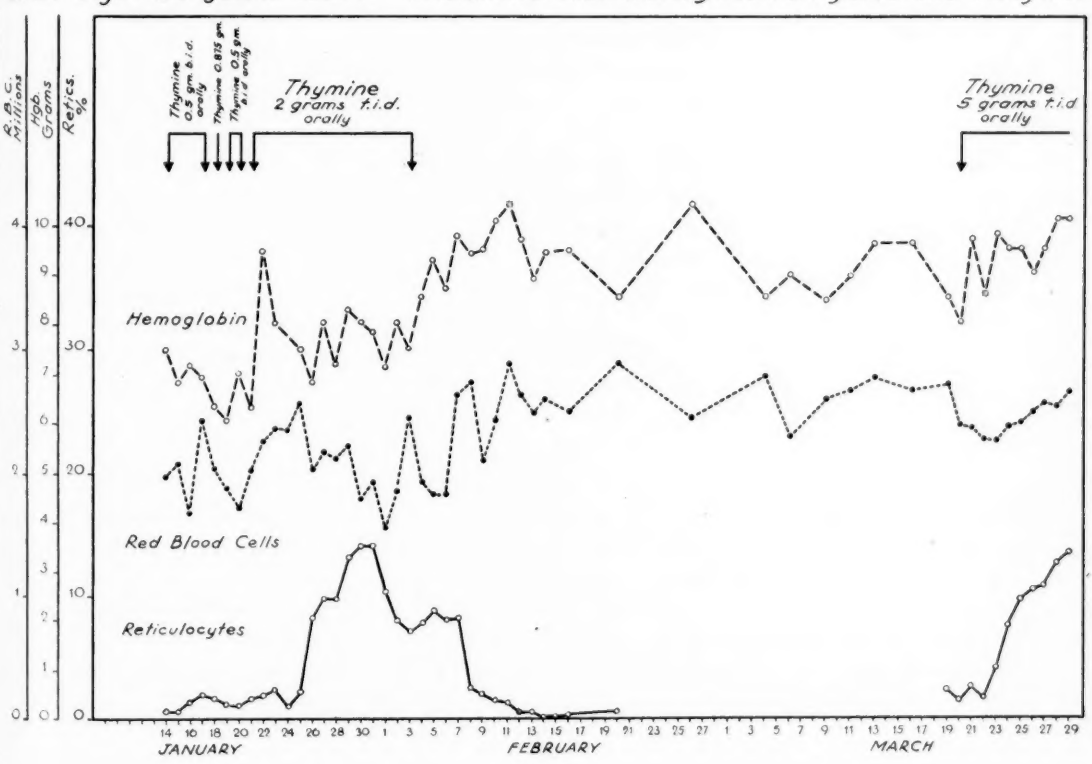

Fig. 1.

The dose was then increased to 2 Gm. t.i.d. On the sixth day after treatment with the larger dose had been instituted, the reticulocytes increased to 8.2 per cent, and a peak reticulocytosis of 14.2 per cent was reached on the eleventh day. Treatment was discontinued after twelve days, but the erythrocytes and hemoglobin continued to rise to a level of 2.88 million and 10.4 Gm. (68 per cent), respectively, on the twenty-second day after treatment with the larger dose was instituted. This was a net increase in erythrocytes of 870,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 4.1 Gm.

within a period of twenty-two days. Sternal bone marrow preparations made at this time showed a reversion toward normal. The patient was then discharged from the hospital and subsequently was observed on several occasions in the Nutrition Clinic. Blood studies were performed biweekly, and it was observed that the erythrocyte level and hemoglobin remained essentially constant until the week prior to readmission to the hospital.

Forty-five days after the patient had received her last dose of 5-methyl uracil, she was readmitted to the hospital for further study. In an effort to reproduce the hematologic response we had initially observed after administration of large doses of 5-methyl uracil in this patient, we resumed treatment, giving the patient 5 Gm. of 5-methyl uracil b.i.d. orally. A reticulocytosis of 4.0 per cent occurred on the fourth day of therapy, and a peak reticulocytosis of 13.2 per cent was reached on the tenth day after treatment had been resumed. On the eleventh day after the present therapy had been instituted, there had been a total increase in erythrocytes of 610,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 2.2 Gm. These results are shown graphically in Fig. 1.

Case 2. Production of a Maximal Hematologic Response to 5-Methyl Uracil.—In an effort to produce the expected maximal hematologic response as calculated by Minot and associates,[4] this patient was given larger doses of 5-methyl uracil than Case 1 received initially. Such a response was obtained. The patient received 3.4 Gm. of 5-methyl uracil t.i.d. orally for eleven days. On the fourth day of therapy the reticulocyte concentration was 6.0 per cent, and a peak reticulocytosis of 19.0 per cent was reached on the sixth day of treatment. On the seventeenth day after therapy was instituted there had been a total increase in erythrocytes of 930,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 3.6 Gm. Sternal bone marrow obtained on the seventh day of therapy showed regeneration as evidenced by a marked increase in normoblasts and a reduction of megaloblasts. Treatment was discontinued for a period of seventeen days and then resumed, the patient receiving thereafter 5 Gm. of 5-methyl uracil b.i.d. On the fifth day of this second course of treatment the reticulocytes were 5.8 per cent, and a peak reticulocytosis of 14.2 per cent was attained on the eighth day after treatment was instituted. By the thirteenth day the erythrocytes had increased by 490,000 per cubic centimeter of peripheral blood, while the hemoglobin remained essentially unchanged. Sternal bone marrow preparations made at the end of the period of therapy showed a reversion toward normal.

Case 3. Determination of a Minimal Dose of 5-Methyl Uracil Which Will Give a Hematologic Response.—In an effort to determine the minimal dose of 5-methyl uracil necessary to effect a hematologic response within a period of two weeks, and in order to determine the minimal dose required for a full hematologic response by employing the double reticulocyte response described by Minot and Castle,[5] this patient was given 1.5 Gm. of 5-methyl uracil t.i.d. orally for a total of thirteen days. A reticulocyte response which appeared to be submaximal occurred, as shown in Table II. Sternal bone marrow obtained on the twelfth day of treatment showed evidence of blood regeneration as well as some megalo-

blastic arrest. This was interpreted as being indicative of a response to inadequate dosage. After the reticulocyte concentration began to fall, the dosage of 5-methyl uracil was increased to 3.4 Gm. t.i.d. orally for a period of five days. A second reticulocyte response occurred, a peak of 8.8 per cent being reached on the sixth day after treatment with this larger dose was instituted. On the twentieth day after therapy with 1.5 Gm. of 5-methyl uracil t.i.d. was instituted, there had been an increase in erythrocytes of 620,000 per cubic centimeter of peripheral blood and an increase in hemoglobin of 3.4 Gm. Sternal bone marrow obtained at this time was normoblastic and in the phase of active regeneration. The patient received no medication for a period of five days following therapy with the larger dose, and he was then given 5 Gm. of 5-methyl uracil b.i.d. as a maintenance dose. A slight but significant reticulocyte response occurred after treatment was resumed. Thus, it is seen that an oral dose of 5-methyl uracil as small as 4.5 Gm. daily will effect a hematologic response in a patient with pernicious anemia in relapse. It is further observed that 4.5 Gm. of 5-methyl uracil daily is an inadequate dose as evidenced by the subsequent reticulocyte response to 10.2 Gm. of 5-methyl uracil daily and also by the presence of an abnormal number of megaloblasts seen in the sternal bone marrow preparation obtained at the end of the course of treatment with this smaller dose.

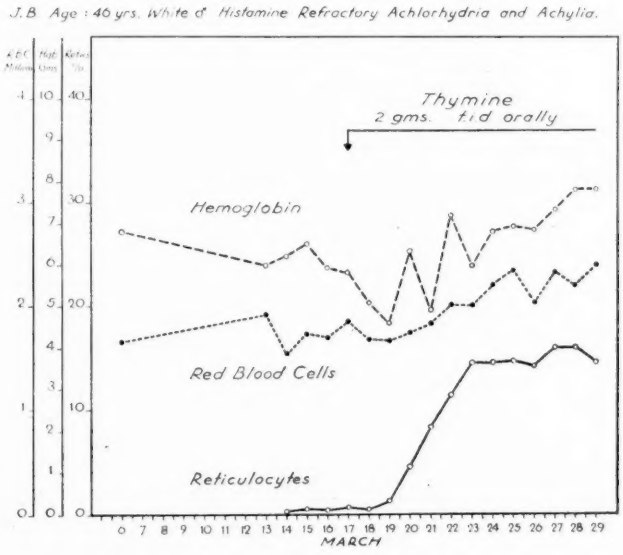

Fig. 2.

Case 4. Hematologic Response to a Suboptimal Dose of 5-Methyl Uracil.—In an effort to determine the minimal dose of 5-methyl uracil required to produce the full calculated hematologic response, we gave this patient what we now believe to be a suboptimal dose. Two grams of 5-methyl uracil were given orally t.i.d. for a period of fourteen days. On the fourth day of treatment the reticulocyte concentration was 4.6 per cent, and a peak reticulocytosis of 16 per cent

was reached on the eleventh day of treatment. The blood findings in this patient are shown in Fig. 2. Sternal bone marrow obtained on the fourteenth day of treatment was normoblastic and without megaloblasts. It is thus seen that a daily oral dose of 2 Gm. of 5-methyl uracil t.i.d. will effect a profound, although not a maximal, hematologic response when given to a patient with pernicious anemia in relapse. Further studies are now being conducted on this patient in an effort to determine the minimal dose of 5-methyl uracil required for a calculated maximal hematologic response.

Case 5. The Hematologic Response to a Large Dose of 5-Methyl Uracil.—This patient received 4 Gm. of 5-methyl uracil t.i.d. orally for a period of eight days. On the third day of treatment the reticulocytes were 3.6 per cent, and a peak reticulocytosis of 31.2 per cent occurred on the seventh day of treatment. Sternal bone marrow obtained after treatment showed great numbers of normoblasts and a substantial reduction in the number of megaloblasts which was indicative of response to an adequate therapeutic dose.

Case 6. Response to 6 Gm. of 5-Methyl Uracil Given Daily.—This patient had a reticulocyte level of from 3.0 to 4.0 per cent over a period of six days during base line observations. Because of the constancy of this reticulocyte level, we felt that it was normal in this individual and that we were not dealing with a so-called spontaneous remission. Sternal bone marrow preparation studies prior to treatment showed megaloblastic arrest and no evidence of regeneration. The patient received 2 Gm. of 5-methyl uracil t.i.d. orally for a period of twelve days. On the second day of treatment the reticulocytes had reached a level of 5.8 per cent, and a peak reticulocytosis of 14.4 per cent occurred on the fifth day of treatment. Sternal bone marrow obtained after treatment was normoblastic and without megaloblasts. It is probably incorrect to state that this patient responded to treatment as early as the second day. However, we believe it is correct to say that this patient responded hematologically to large doses of 5-methyl uracil.

DISCUSSION

The scientific investigation of pernicious anemia dates from the time of Thomas Addison when, in 1855, he described the anemia which now bears his name. A milestone in these investigations was passed in 1926, when Minot and Murphy[6] effected a hematologic remission in patients with pernicious anemia by feeding them large amounts of liver by mouth. Not until the fall of 1945, when synthetic folic acid was shown to have antianemia effects in patients with macrocytic hyperchromic anemia,[3, 7] were investigators able to use pure synthetic compounds in their studies. The studies on folic acid have been extended, and the efficacy of this substance in the treatment of pernicious anemia, nutritional macrocytic anemia, and the anemia of sprue has been established.[8-12] The observation that 5-methyl uracil will produce a hematologic remission in patients with pernicious anemia in relapse necessitates further studies of the functional relationship between folic acid and 5-methyl uracil. We realize that folic acid may act as an enzyme or as a coenzyme in the synthesis of 5-methyl uracil or of substances which act similarly. Studies are likewise indicated as to the efficacy

of synthetic 5-methyl uracil in the treatment of nutritional macrocytic anemia and the anemia of sprue. This compound will probably prove as effective in the treatment of these two types of macrocytic hyperchromic anemia as in the treatment of pernicious anemia in relapse.

SUMMARY AND CONCLUSION

Six patients with Addisonian pernicious anemia in relapse were selected for study. They were hospitalized and given a diet devoid of meat, meat products, fish, poultry, and raw vegetables. After an initial period of base line studies, synthetic 5-methyl uracil was administered in varying doses by mouth to each patient. Six patients are reported in whom a hematologic response was produced by the daily oral administration of synthetic 5-methyl uracil when given in doses of 4.5 Gm. or more. Case 1 is of special interest in that the hematologic response to large doses of 5-methyl uracil was reduplicated after a period of forty-five days in which no therapy was given. Case 2 shows the production of calculated maximal hematologic response after the daily oral administration of 10.2 Gm. of 5-methyl uracil.

It is concluded that synthetic 5-methyl uracil is a potent antianemic substance when given orally in daily doses of 4.5 Gm. or more to patients with pernicious anemia in relapse.

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EFFECT OF PENICILLIN AND SULFONAMIDES ON ACETYLCHOLINE SYNTHESIS AND ON THE RESPONSE OF MUSCLE TO ACETYLCHOLINE AND POTASSIUM*

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A RECENTLY presented concept concerning the pathogenesis of myasthenia gravis suggests that there is a defect in either the activity or the amount of enzyme that synthesizes acetylcholine.[1-3] Since patients with myasthenia gravis may in addition suffer from infectious diseases, some of which are treated with penicillin and sulfonamides, it becomes pertinent to investigate the effect of penicillin and sulfonamides on acetylcholine synthesis.

EXPERIMENTAL

Effect of the Substances on Acetylcholine Synthesis.—The effect of penicillin and sulfonamides on the acetylcholine synthesis was studied by a method described previously.[3] Mixtures containing varying amounts of these drugs, 100 mg. of minced fresh frog brain, 3 mg. of physostigmine salicylate, and 3 c.c. of Ringer's solution, were shaken and incubated aerobically for four hours at 37° C. After incubation the amounts of acetylcholine synthesized were assayed biologically on the sensitized rectus abdominis muscle of the frog.

Calculation: The amount of acetylcholine synthesized was calculated by subtracting from the acetylcholine content of the incubated mixtures the acetylcholine content of identical nonincubated mixtures. The amount of acetylcholine synthesized in the control mixtures containing only brain, physostigmine, and Ringer's solution was taken as 100 per cent. The acetylcholine content of the mixtures containing penicillin and sulfonamides was expressed as a per cent of the control.

The results are given in Table I. The sulfonamides studied did not modify the acetylcholine synthesis. Penicillin, however, even in relatively low concentrations, decreased the acetylcholine synthesis.[4]

Effect of the Substances on the Response of Striated Muscle to Acetylcholine.—Acetylcholine participates in the humoral transmission of the effects of stimulation of the motor nerve to the striated muscle. There are many substances known to increase the response of the muscle to acetylcholine, and many of them act by inhibiting the hydrolysis of acetylcholine by decreasing the activity of cholinesterase.

In the following experiments the effect of penicillin and sulfonamides on the response of the muscle to acetylcholine was investigated, following a method described previously.[5] The rectus abdominis muscle of the frog was excised

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TABLE I. EFFECT OF SULFONAMIDES AND PENICILLIN ON ACETYLCHOLINE SYNTHESIS

SUBSTANCE	AMOUNT OF ACETYLCHOLINE SYNTHESIZED IN PER CENT OF CONTROL*								
	Amount of Sulfonamides (mg.) Added to 100 mg. Frog Brain								
	3		0.3		0.03		0.003		
Sulfanilamide	113		117		114		107		
Sulfapyridine	109		106		104		105		
Sulfathiazole	100		106		107		104		
Sulfadiazine	99		100		96		97		
	Amount of Penicillin (Oxford Units) Added to 100 mg. Frog Brain								
	30,000	15,000	3,000	1,500	300	150	30	15	3
Penicillin	6	15	35	50	68	90	92	94	99

*Each value represents the average of ten separate experiments. The standard error of the mean for each value was less than ±5 per cent.

and suspended in a muscle chamber containing 10 c.c. of Ringer's solution. Shortening of the muscle was induced by immersion in a Ringer's solution containing 50 μg acetylcholine bromide per 100 c.c. for two minutes. The shortening of the muscle was registered by an isotonic lever on a kymograph. The muscle was then washed with Ringer's solution for ten minutes and shortened again with the acetylcholine solution. This procedure was repeated until three successive immersions in the acetylcholine solution gave similar responses. Then between two shortenings induced by the acetylcholine solution, instead of washing in Ringer's solution for ten minutes, the muscle was washed for only five minutes and immersed in a series of Ringer's solutions containing either penicillin or one of the sulfonamides, in increasing concentrations, for five minutes. The muscle shortening after immersion in the solutions of the drugs was expressed as percentage of the shortening of the same muscle before immersion in the solutions of the drugs. The shortening induced by acetylcholine in control muscles immersed only in Ringer's solution remained unchanged for at least three hours. This period of time was longer than the duration of the experiments described. Since the standard error of the mean for each experiment was less than ±4 per cent, all results deviating from 100 per cent by more than 12 per cent are probably significant deviations:

$$(2\sqrt{\text{S.E.}^2\ \text{(control)} + \text{S.E.}^2\ \text{(experiments)}} = 2\sqrt{4^2 + 4^2} = 11.5)$$

The results are given in Table II. Penicillin, in the concentrations used, did not modify the response of the muscle to acetylcholine; sulfonamides, in relatively high concentrations, slightly increased the response of the muscle to acetylcholine.

Effect of Penicillin and Sulfonamides on the Response of Striated Muscle to Potassium.—To ascertain whether the drugs used modify the response of the striated muscle to chemical stimuli other than acetylcholine, the effect of the drugs on the response of the muscle to potassium was also investigated. Potassium was chosen to induce shortening of the muscle, since the effect of potassium usually parallels the effect of direct stimulation of the muscle.[6, 7]

The rectus abdominis muscle of the frog was prepared as previously described. Muscle shortening was induced by immersion in a 20 mM KCl solution instead of an acetylcholine solution. The effect of penicillin and sulfonamides

TABLE II. EFFECT OF SULFONAMIDES AND PENICILLIN ON CONTRACTION OF RECTUS ABDOMINIS MUSCLE INDUCED BY ACETYLCHOLINE AND POTASSIUM

SUBSTANCE	MAGNITUDE OF CONTRACTION IN PER CENT OF CONTROL;* CONTRACTION INDUCED WITH											
	ACETYLCHOLINE						POTASSIUM					
	Concentration (Mg.) of the Sulfonamides Per 100 c.c. Ringer's Solution											
	100	10	1	0.1	0.01	0.001	100	10	1	0.1	0.01	0.001
Sulfanilamide	113	102	100	99	100	101	63	86	108	101	100	100
Sulfapyridine	123	102	102	99	98	101	66	79	102	102	100	99
Sulfathiazole	139	111	100	103	101	102	50	76	93	100	102	100
Sulfadiazine	116	100	98	97	99	100	69	78	100	99	99	100
	Concentration (Oxford Units) of Penicillin in 100 c.c. Ringer's Solution											
	200,000	100,000	20,000	10,000	1,000	100	200,000	100,000	20,000	10,000	2,000	1,000	100
Penicillin	96	94	95	98	98	99	230	195	145	138	120	115	100

*Each value represents the average of ten separate experiments. The standard error of the mean for each value was less than ±4 per cent.

was ascertained by immersion of the muscle in a series of solutions containing the drugs in increasing concentrations, for five minutes, before immersion in the KCl solution, for two minutes.

The results are given in Table II. Penicillin, in higher concentrations, increased the potassium sensitivity of the muscle, and the sulfonamides, in higher concentrations, decreased the response of the muscle to potassium.

DISCUSSION

Penicillin decreased the amount of acetylcholine synthesized probably because it reduces the activity of the –SH group,[8] an active group contained in the enzyme involved in the synthesis of acetylcholine.[9]

Since penicillin did not modify the response of the muscle to acetylcholine, it is probable that penicillin, in the concentrations used, does not inhibit cholinesterase. The effect of penicillin in increasing the potassium sensitivity of the muscle suggests that penicillin may sensitize the muscle to direct stimulation.

The sulfonamides, in the concentrations used, did not modify the amount of acetylcholine synthesized. In relatively high concentrations, sulfonamides increased the response of the muscle to acetylcholine, suggesting that they may inhibit cholinesterase in high concentrations. Sulfonamides apparently do not increase the sensitivity of the muscle to direct stimulation.

SUMMARY

1. The effect of penicillin and sulfonamides on acetylcholine synthesis and on the response of the striated muscle to chemical stimuli were investigated.

2. Penicillin, in relatively low concentrations, decreased the acetylcholine synthesis. The sulfonamides did not modify the synthesis of acetylcholine.

3. In the concentrations used, penicillin did not increase the acetylcholine sensitivity of the muscle. Sulfonamides, in higher concentrations, slightly increased the acetylcholine sensitivity of the striated muscle.

4. Penicillin increased the potassium sensitivity of the muscle suggesting that it may render effector cells hypersensitive to direct stimulation. Sulfonamides, in higher concentrations, decreased the potassium sensitivity of the striated muscle.

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THE ANTISHOCK ACTION OF ETHANOL IN BURNED MICE

Effect on Edema Formation and Capillary Atony

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IN PREVIOUS reports from this laboratory, it was shown that the administration of 15-unit liver extract increased survival time and diminished mortality in rats and mice subjected to a standardized shock-producing scalding procedure.[1, 2] The extract was found to be effective if administered prior to scalding, an observation which has been confirmed by Haterius and Glassco,[3] but not if given after the burn.

During the testing of various fractions derived from 15-unit liver extract, it was observed that one particular fraction, an ethanol extract, was especially active in counteracting shock.* In an effort to purify this fraction, which contained 25 Gm. of ethanol per 100 c.c., it was found that the distillate carried all the antishock activity, whereas none was exhibited by the residue. When, however, ethanol-free 15-unit liver extract was distilled, the residue retained all the activity and the distillate was inactive. In this manner it was shown that the ethanol containing distillate of the special fraction had antishock activity similar to that of ethanol-free liver extract. This led to a study of whether ethanol itself had a preventive effect upon the development of burn shock.

In recent experiments in this laboratory,[4] it was found that two separate factors were operative in the causation of scald shock, in each instance by reducing the circulating blood volume; the one, local fluid loss, considered by many to play a major role in the initiation of burn shock[5] and the other, capillary atony, leading to stagnation of blood in the visceral organs.[6] In scalded animals, it was found that the amount of blood retained in the visceral capillaries was significantly greater than in controls. By experiment it was ascertained that the most satisfactory method of demonstrating blood retention was to make comparative counts of open capillaries and estimations of the hemoglobin content of the viscera, but only after exsanguinating the completely anesthetized animals. In shock, removal from the active circulation of large amounts of blood trapped in atonic vessels in the capillary bed contributes to the observed reduction in the circulatory volume. The capillary atony is believed to be caused by a toxic factor which was demonstrated in the blood of scalded animals by transfusion[4] and renal perfusion[7] experiments.

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The relative importance of local fluid loss and the capillary toxic factor in reducing the circulatory volume and initiating shock varied with the character of the burn and the time interval after thermal trauma.

The purpose of this communication is (1) to bring forward evidence that ethanol, like 15-unit liver extract, increases survival time and diminishes mortality in mice subjected to scald shock and (2) to report studies on the mechanism whereby ethanol retards the development of shock.

METHODS

All experiments were performed on Swiss mice weighing from 16 to 26 grams. For any particular experiment, the mice obtained from a single source were of a uniform size. The method of scalding and the details of evaluating results have been reported in a previous communication.[2] Ethanol in various concentrations was injected intraperitoneally in 0.2 c.c. of physiologic salt solution, an amount which has no measurable antishock activity.[2] The bleeding volume was ascertained by cutting out the heart of the anesthetized animal and mopping up the blood from the thoracic cavity with a weighed cotton pledget. The amount of fluid loss into scalded limbs was determined by a modification of Blalock's bisection technique.[8] All the animals used in these experiments were completely anesthetized with ether immediately prior to scalding. The method of estimating hemoglobin retention in the tissues is given under the appropriate experiment.

Effect of Ethanol on Survival Time and Mortality in Burn Shock.—Twelve groups of at least twenty mice each were injected intraperitoneally with 25 per cent ethanol (0.2 c.c. per mouse) from thirty to forty minutes before scalding to the head at 65° C. for ten seconds. The responses to the thermal injury were compared with those in twelve parallel groups injected with 0.2 c.c. of isotonic salt solution. As shown in Table I, Experiment C, ethanol significantly prolonged survival time and lessened mortality in scalded mice.

The effect of concentration of ethanol was investigated in the same manner. It was found that 0.2 c.c. of 15 per cent ethanol produced a significant increase in survival time and a decrease in mortality, but the effect was not quite as great as that obtained with a 25 per cent solution. The injection of 0.2 c.c. of 5 per cent ethanol was without measurable activity. The results are presented in Table I, Experiments C, D, and E.

These experiments show that ethanol administered prior to scalding displays antishock activity similar to that noted in previous studies with ethanol-free 15-unit liver extract.[1, 2] That the observed effects on survival time and mortality rate are not due to an intraperitoneal injection itself is proved by the negative results obtained in control experiments with 0.2 c.c. of isotonic salt solution injected intraperitoneally and with a variety of other substances tested in previous studies.[9] To ascertain the effect of a peritoneal irritant, tincture of catharides was injected intraperitoneally into mice prior to scalding. The results of this experiment were negative.

Effect of Time of Injection on Antishock Activity of Ethanol.—In Table I, data are presented on the protective activity of 0.2 c.c. of 25 per cent ethanol

TABLE I. EFFECT OF ETHANOL ON SURVIVAL TIME AND MORTALITY IN BURN SHOCK

EXPERIMENT	PER CENT ETHANOL (GM. PER 100 GM.)	TIME OF INJECTION IN RELATION TO BURN	NUMBER OF TRIALS	TOTAL NUMBER OF MICE	PER CENT INCREASE IN AVERAGE SURVIVAL TIME OVER CONTROLS		P† VALUE	INCREASE IN PER CENT OF SURVIVORS OVER CONTROLS		P† VALUE
					MEAN	S.E.*		MEAN	S.E.*	
A	25	18 hr. before	5	245	47	22	> 0.1	12	5	< 0.05
B	25	5 hr. before	1	38	182			25		
C	25	30 min. before	12	515	164	38	< 0.001	55	6	< 0.001
D	15	30 min. before	5	207	139	25	< 0.01	20	5	< 0.01
E	5	30 min. before	5	207	20	21	> 0.8	4	2	> 0.2
F	25	5 min. before	4	198	155	25	< 0.02	26	11	< 0.01
G	25	1 min. before	2	95	46			0		
H	25	1 min. after	2	93	−52			−6		
I	25	5 min. after	3	137	−25			−4		
J	25	30 to 60 min. after	5	212	− 2	23	> 0.9	−4	3	> 0.9
K	No injection		17	772	15	6		0	2	

*Standard error of the mean $\left(= \frac{\text{Standard deviation}}{\sqrt{n}} \right)$.

†In each case comparison is made with Experiment K. P value was estimated by the method of Fisher[14] from the t value

$$\left(t = \frac{\text{Difference of the means}}{\text{Standard deviation of the differences of the treated and Experiment K groups}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}\right);$$

n_1, n_2 = number of trials in the groups compared. P value of less than 0.05 indicates a significant difference in response to treatment.

injected at various time intervals prior to scalding. As shown in the table (Experiment A), ethanol injected eighteen hours before the burn resulted in an average increase in survival time of 47 per cent and an average decrease in mortality of 12 per cent. When compared with controls, the average increase in survival time was not within the range of significant activity, although an apparently significant number of mice survived longer than the control mice. On the other hand, ethanol administered between five and thirty minutes before the animals were scalded significantly increased survival time and reduced mortality (Experiments C and F). In a single experiment employing thirty-eight mice, a positive result was obtained with ethanol injected five hours before scalding (Experiment B). No activity was noted when ethanol was injected one minute before scalding, probably because there was insufficient time for absorption (Experiment G). Negative results were obtained with ethanol administered from one to sixty minutes after the burn (Experiments H, I, and J).

Effect of Ethanol on Bleeding Volume in Shock.—An appreciable reduction of the bleeding volume has been demonstrated in burn shock.[10, 11] Since ethanol has prophylactic activity in shock, its influence on the bleeding volume after burns was investigated. As seen in Fig. 1, ethanol did not significantly influence the bleeding volume in unburned mice. Scalded mice given a control injection of 0.2 c.c. of isotonic salt solution showed a marked reduction of the bleeding volume. This reduction was prevented to a significant extent when ethanol was injected thirty minutes prior to scalding. The bleeding volume was unchanged when ethanol was given after the burn.

From these experiments, it is concluded that the prophylactic effect of ethanol is related to its ability to prevent the reduction of bleeding volume which occurs in mice shocked by scalding.

Effect of Ethanol on Local Fluid Loss.—As previously stated, the reduction in the effective circulatory volume in burn shock is believed to be due to two factors, local fluid loss at the site of the trauma and capillary atony.[4] The

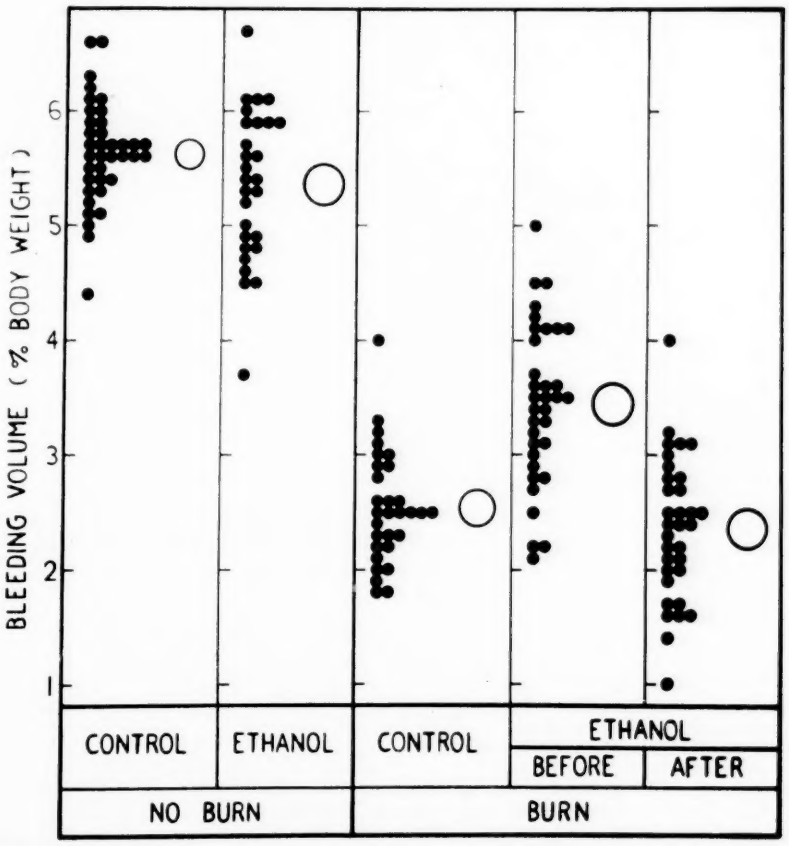

Fig. 1.—Effect of ethanol on bleeding volume in burn shock. Mice were scalded to the head at 65° C. for ten seconds. One group of burned animals was injected with 25 per cent ethanol thirty minutes before and a second group five minutes after, scalding. Unburned, control animals received isotonic salt solution and ethanol, respectively, and burned, control animals received only saline. All solutions were injected intraperitoneally in amounts of 0.2 c.c. Bleeding volumes in the unburned mice were taken from one to two hours after injection of the test solution and in the burned animals at a similar time interval after thermal injury. Each dot represents the response of a single mouse. The large circles represent the mean response; the diameter of each large circle equals three times the standard error of the mean.

antishock properties of ethanol would be expected to result from its ability to diminish local edema formation or capillary atony or both. The following experiment was performed to determine the effect of ethanol on local fluid loss (edema formation).

Fluid loss in the scalded parts was studied by a modification of Blalock's bisection method.[8] One hindlimb of each of seventy-two anesthetized mice

was scalded at 65° C. for ten seconds. Approximately three hours after scalding, each animal was again anesthetized with ether, the bleeding volume determined, and the amount of fluid loss into the tissues ascertained by bisecting the animal, severing the hindquarters on each side, and comparing the weights of the two hindquarters. In observations on twelve unburned mice (Fig. 2), the average difference in weight of the hindquarters in terms of percentage of body weight was 0.39 ± 0.07* per cent, showing that the accuracy of the bisecting technique for mice equaled that for rats.[8]

Estimation of the amount of edema formation was made in two sets of animals, the one receiving 0.2 c.c. of 25 per cent ethanol intraperitoneally prior to scalding and the other receiving 0.2 c.c. of salt solution only. The results as shown in Fig. 2 were as follows: in forty-eight control burned mice, the average fluid shift to the scalded side was 5.02 ± 0.15* per cent; in twenty-four burned mice treated with ethanol, this value was 3.73 ± 0.19* per cent. The difference is statistically significant.

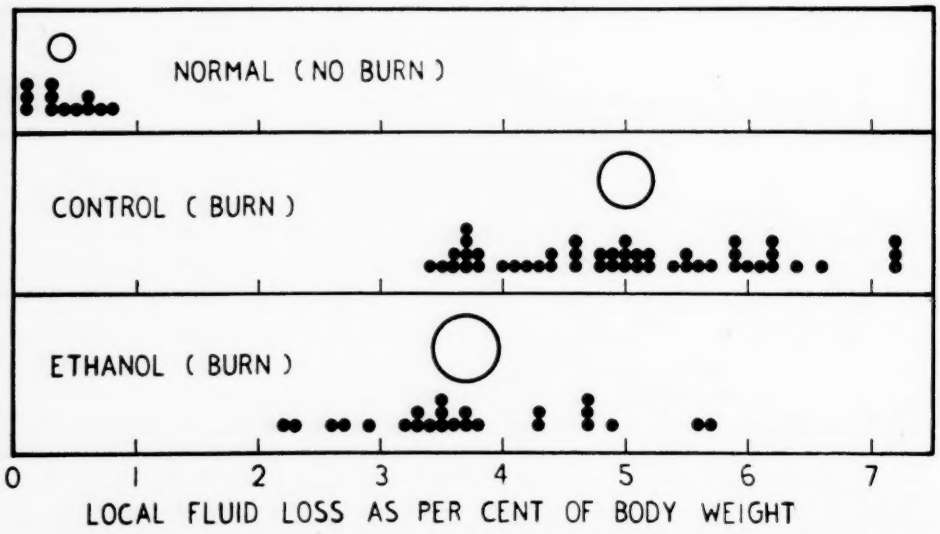

Fig. 2.—Effect of ethanol on local fluid loss. Mice were injected intraperitoneally with 0.2 c.c. of isotonic salt solution and 25 per cent ethanol, respectively, prior to burning a single hindlimb at 65° C. for ten seconds. Estimation of the amount of local fluid loss was made from two to four hours after the burn. Each dot represents the response of a single mouse. The large circles represent the mean response; the diameter of each large circle equals three times the standard error of the mean.

As shown in Fig. 2, the effect of preliminary treatment with ethanol was to reduce significantly the amount of edema in the scalded parts. From previous experience in this laboratory, when one hindlimb was scalded at 65° C. for ten seconds, death did not result within forty-eight hours in a single instance, in spite of the fact that the local fluid loss often amounted to as much as 5 per cent of the body weight.

Prophylactic Effect of Ethanol on Capillary Atony.—As described in previous publication, shock and death can be produced with little or no local fluid

*Standard error of the mean.

loss following a severe burn at 100° C.[4, 8] In this type of thermal trauma, it was demonstrated that the reduction in the circulatory blood volume was largely due to visceral capillary congestion which resulted from the operation of a toxic factor in the blood.[4] The degree of capillary atony was ascertained by measuring the amount of blood retained in an organ, that is, liver, after exsanguination of the animal. Capillary atony also contributes to the production of shock in burns which are accompanied by considerable fluid loss. In mice scalded to the head at 65° C. for ten seconds, the amount of hemoglobin retained in the liver was appreciably greater than in normal controls.[4]

In order to ascertain the effect of ethanol upon capillary atony, the following experiments were performed. Three groups of mice, each consisting of fifty-seven animals, were used; these groups were divided into six duplicate sets of nine or ten mice each. The first group consisted of animals which received an intraperitoneal injection of 0.2 c.c. of 25 per cent ethanol thirty minutes prior to scalding to the head at 65° C. for ten seconds. The second group was scalded in a similar manner but received 0.2 c.c. isotonic salt solution instead of ethanol. The third group consisted of normal, unburned mice which served as controls to correct for the varying degrees of hemoconcentration noted after this type of burn. Bleeding volumes were determined by exsanguination; samples of blood were taken, and the livers were removed for estimation of hemoglobin. In the first two groups, this was done from three to four hours after the burn. The amount of hemoglobin retention in the exsanguinated livers was used as an index of the degree of capillary atony.

Method of Determining Hemoglobin in Liver: After testing published methods and experimenting with modifications, the following procedure was found to be the most satisfactory and expedient for making comparative measurements of the amount of hemoglobin retained in the tissue under investigation. Hemoglobin retained by the liver was extracted with a buffered salt solution and measured photometrically after conversion to cyanmethemoglobin.[12] The liver was finely minced with scissors and covered with a volume of buffered salt solution equal to ten times the weight of the tissue. This buffered salt solution was prepared by dissolving 300 Gm. of sodium chloride, 7.62 Gm. of sodium tetraborate (borax), 6.18 Gm. of boric acid, and 0.50 Gm. of saponin in distilled water, by diluting to 1 L., and by filtering. The pH of this solution varied between 7.05 and 7.15. Extraction with occasional shaking was continued for eighteen hours or more. The mixture was then filtered through Whatman filter paper No. 2. This procedure gave brilliant filtrates, so that there was no need for turbidity corrections. To the filtrate was added one drop of 20 per cent potassium ferricyanide to convert the hemoglobin into methemoglobin; the latter was converted to cyanmethemoglobin by the addition of one drop of a freshly prepared mixture of equal parts of 10 per cent sodium cyanide and 12 per cent acetic acid. The cyanmethemoglobin color was estimated with the Klett-Summerson photoelectric colorimeter, using Filter 54.

A few facts concerning the practical application of this and other methods are pertinent. Liver minced with scissors and leached for eighteen hours by the method described does not yield all of its color at the end of this period, since leaching of the residue left by filtration for an additional eighteen hours yields an added 10 per cent of cyanmethemoglobin color. Nevertheless, leaching for an eighteen-hour period does provide satisfactory and uniform values when comparative observations are made. Tests performed in this manner, with like amounts of tissue taken from the same organ, gave hemoglobin values within 5 per cent of each other. This method was found suitable for making comparative

estimations of hemoglobin in blood and in tissue other than liver. The use of the Waring blender for mincing the liver was found to be unsatisfactory; low recovery values for hemoglobin were obtained, because most of the pigment was absorbed by the colloidal mass. Methods designed to recover hemoglobin as iron by wet ashing the tissue were tedious and difficult to evaluate due to the presence of variable amounts of nonhemoglobin iron.

As shown in Table II, 91 per cent more hemoglobin was retained in the liver of the saline-treated scalded mice than in the unburned controls and only 40 per cent more in the ethanol-treated animals. Statistical analysis shows that the difference between these figures is significant. Thus, the administration of ethanol significantly reduced the amount of hemoglobin usually retained in the liver after scalding.

TABLE II. PROPHYLACTIC EFFECT OF ETHANOL ON HEMOGLOBIN RETENTION IN BURN SHOCK

EXPERIMENT	NUMBER OF LIVERS COMPARED IN EACH GROUP	HEMOGLOBIN RETENTION AS PER CENT OF VALUE IN UNBURNED, EXSANGUINATED GROUP	
		ETHANOL (BURNED)	SALINE (BURNED)
1	10	137	218
2	9	116	204
3	10	145	216
4	9	145	143
5	9	125	141
6	10	174	216
Mean ± S.E.*		140 ± 8	191 ± 15
P value*		< 0.05	

*Symbols and method of calculation same as Table I.

These observations confirm previous ones, that scalding leads to visceral congestion (increase in hemoglobin retention) in the liver, and lead to the conclusion that ethanol administered in the manner described owes its antishock action in part to a reduction in visceral capillary atony.

DISCUSSION

By employing a standard procedure for producing burn shock in mice, which consists of scalding the anesthetized animals to the head at 65° C. for ten seconds, it was shown that the intraperitoneal injection of ethanol prior to scalding increased the average survival time and diminished the average mortality rate. The results with ethanol parallel those previously obtained with ethanol-free 15-unit liver extract. Ethanol, like liver extract, had no antishock activity when administered after the thermal injury.

In experiments reported in this and in previous communications, it was shown that when mice were scalded in the manner described the bleeding volume in the shocked animals was significantly reduced. A decreased bleeding volume has been accepted as an index of reduced circulating blood volume, and it is generally agreed that the reduction in the circulatory volume is largely responsible for the chain of events which leads to shock in burns. It was shown that ethanol, when injected in suitable concentration *prior* to scalding, curtailed the usual reduction of bleeding volume, and this fact explains the increase in survival time and diminution in mortality. On the other hand, the reduction in bleeding volume in scalded mice was not influenced

when ethanol was given after the burn, a fact which explains the failure of ethanol to protect the animals when this substance was administered after the trauma.

Two factors have been found to be responsible for the reduced circulating volume in burn shock; the one, fluid loss at the site of trauma and the other, capillary atony, due to a toxic agent.[4] The influence of ethanol on both of these factors was investigated. By comparing the amount of local fluid loss in burned mice given ethanol with that in control, burned animals, it was shown that ethanol curtailed the amount of fluid lost in a burned limb when this substance was injected prior to scalding but not when administered after the injury.

The mechanism whereby ethanol inhibits local fluid loss or edema formation requires further investigation. In a companion paper, data are presented which show that barbiturates, as well as other substances, have antishock properties paralleling those of ethanol and that the prophylactic effects are due in part to the curtailment of edema formation. A discussion of the factors which may be responsible for the observed inhibitory effects upon local fluid loss will be found in that paper.[13]

There remains for discussion, in considering the mechanism of the antishock action of ethanol, the effect of this substance upon the operation of a toxic factor. In previous experiments performed in this laboratory,[4] it was shown that the pooling of large amounts of blood in atonic visceral capillaries contributes to the fall in the circulatory volume and that this congestion is due to a circulating toxic factor.

The degree of visceral congestion was estimated by comparing the amount of blood retained in the liver of burned, exsanguinated mice with that in unburned controls. In scalded mice there was a notable retention of blood in the hepatic capillaries. Experiments showed that the prophylactic administration of ethanol resulted in a significant reduction of the amount of blood retained in the liver of scalded animals but did not influence the amount of blood retained in the liver of unburned controls. The manner in which ethanol counteracts capillary atony is not known. It is possible that this substance neutralizes the hypothetic humoral toxic factor which causes capillary atony, or it may decrease the responsiveness of the capillaries to this factor.

SUMMARY AND CONCLUSIONS

Intraperitoneal injection of ethanol increases survival time and diminishes mortality in mice subjected to a standardized thermal injury. This agent has a prophylactic effect when administered before the burn but is without antishock action when given after the trauma.

In burn shock, the circulating blood volume as represented by the bleeding volume is significantly reduced, and the prophylactic administration of ethanol curtails the expected reduction. Ethanol counteracts shock (1) by diminishing local fluid loss (edema formation) and (2) by reducing visceral congestion due to capillary atony.

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THE ANTISHOCK ACTION OF CERTAIN DRUGS IN BURNED MICE

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IN A companion paper,[1] we reported that ethanol increased survival time and diminished mortality in mice subjected to a standardized shock-producing scalding procedure. In order to be effective as an antishock agent, it was necessary to administer ethanol before the burn, for it had no therapeutic or prophylactic action when given after the trauma.

In previous studies, it was shown that two major factors contributed to the initiation of scald shock; the one, fluid loss at the site of thermal injury and the other, visceral congestion due to capillary atony.[2] Each of these factors in suitable degree, separately or in combination, leads to a reduction in the circulating blood volume.

In order to investigate the mechanism whereby ethanol exerts its antishock action, studies were made to ascertain the effect in burned animals of prophylactic administration of this agent upon the circulatory volume, the amount of local fluid loss, and the degree of visceral congestion. From these experiments, it was concluded that ethanol counteracts shock by curtailing local fluid loss and by reducing visceral congestion.

In a previous report, it was shown that ethanol-free 15-unit liver extract displays the same kind of antishock action as was subsequently demonstrated for ethanol.[3] The purpose of the present communication is to report investigations upon compounds related either chemically or pharmacologically to ethanol. In these studies, similar antishock activity was demonstrated for sodium pentobarbital, morphine, histamine, acetone, glycerol, and propylene glycol. These substances were selected for the following reasons: glycerol and propylene glycol because they are alcohols; acetone because of the similarity of its pharmacologic action to that of ethanol; sodium pentobarbital and morphine because of their narcotic properties; and histamine because of its vasodilator activity. The mechanism of their action was investigated by the methods described in the companion paper on ethanol.[1]

EXPERIMENTS AND RESULTS

Effect of Various Drugs on Survival Time and Mortality in Scald Shock.—Glycerol and Propylene Glycol: In six trials, in which glycerol (25 per cent) was injected intraperitoneally into mice thirty minutes prior to scalding to the head at 65° C. for ten seconds, there was a significant increase in survival time and in the number of survivors during the forty-eight hour period

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TABLE I. EFFECT OF VARIOUS DRUGS ON SURVIVAL TIME AND MORTALITY IN BURN SHOCK

EXPERIMENT	DRUG	AMOUNT INJECTED BEFORE BURN	NUMBER OF TRIALS	TOTAL NUMBER OF MICE	PER CENT INCREASE IN AVERAGE SURVIVAL TIME OVER CONTROLS		P† VALUE	INCREASE IN PER CENT SURVIVORS OVER CONTROLS		P† VALUE
					MEAN	S.E.*		MEAN	S.E.*	
A	Glycerol	0.2 c.c. of 25%	6	252	123	43	< 0.02	11	6	< 0.05
B	Propylene glycol	0.2 c.c. of 25%	6	255	92	28	< 0.02	12	5	< 0.05
C	Acetone	0.2 c.c. of 25%	5	212	169	55	< 0.01	23	7	< 0.01
D	Sodium pentobarbital	1 mg. in 0.2 c.c.	7	280	255	70	< 0.01	20	7	< 0.01
E	Morphine sulfate	0.5 mg. in 0.2 c.c.	7	280	371	44	< 0.01	30	7	< 0.01
F	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	7	280	326	53	< 0.01	20	2	< 0.01
G	No injection		17	772	15	6		0	2	

*Standard error of the mean $\left(= \frac{\text{Standard deviation}}{\sqrt{n}}\right)$.

†In each case comparison is made with Experiment G. P value was estimated by the method of Fisher[16] from the t value.

$\left(t = \frac{\text{Difference of the means}}{\text{Standard deviation of the differences of the treated and Experiment G groups}} \sqrt{\frac{n_1 n_2}{n_1 + n_2}}\right)$;

n_1, n_2 = number of trials in the groups compared. P value of less than 0.05 indicates a significant difference in response to treatment.

of observation (Table I, Experiment A). Similar results were obtained with propylene glycol (25 per cent), as shown in Table I, Experiment B.

Acetone: In five trials, with a 25 per cent solution, it was found that acetone was as effective as ethanol in diminishing mortality and increasing survival time (Table I, Experiment C).

Sodium Pentobarbital: In seven trials, with the prophylactic injection of 1 mg. in 0.2 c.c. of physiologic salt solution, there was a significant increase in survival time and in the number of survivors (Table I, Experiment D). Sodium pentobarbital in the dosage used exhibited a greater narcotic effect than 0.2 c.c. of a 25 per cent solution of ethanol.

Morphine: Morphine sulfate (0.5 mg. in 0.2 c.c. of physiologic salt solution) was injected intraperitoneally from thirty to forty minutes prior to scalding. In seven trials, the results showed a significant reduction in mortality and an increase in survival time (Table I, Experiment E). The dose of morphine administered was sufficient to maintain the curved-tail response[4] for the period prior to scalding.

Histamine: Seven trials were performed in which histamine diphosphate (0.1 mg. as histamine base in 0.2 c.c. of physiologic saline) was injected intraperitoneally into each mouse from five to eight minutes prior to scalding. This short interval was chosen because the vasodilator action of histamine is comparatively brief. The results showed that histamine increased survival time and decreased mortality to a significant degree (Table I, Experiment F).

Effect of Various Drugs on Local Fluid Loss and Bleeding Volume in Scald Shock.—A major factor which contributes to the reduction in the circulatory blood volume in burn shock is fluid loss or edema at the site of the trauma. Ethanol administered prior to scalding was found to curtail the usual reduction in the circulating volume and to diminish the amount of local edema formation.[1]

Effect on Local Fluid Loss and Bleeding Volume of Drugs Administered Before Burning: In order to investigate the effect of morphine, sodium pentobarbital, and histamine upon local fluid loss and the circulatory volume in burn shock, the following experiments were performed. One hind limb of each of 120 anesthetized mice was scalded at 65° C. for ten seconds. Approximately three hours after scalding, each animal again was anesthetized with ether, the bleeding volume determined, and the amount of fluid loss into the tissue ascertained by the method previously described.[5] Estimation of the amount of local edema formation was made on four groups of burned animals, all of which had been given a test substance intraperitoneally thirty minutes prior to scalding. The first group was given morphine sulfate; the second, sodium pentobarbital; the third, histamine; and the fourth, a control injection of physiologic salt solution.

As shown in Table II, the effect of prophylactic treatment with morphine, sodium pentobarbital, and histamine was to reduce significantly the amount of edema formation in the burned limb. The expected diminution of the bleeding volume was prevented to a significant extent. It should be noted, however, that in this experiment in which only one limb was burned the reduction in bleeding volume induced by the thermal trauma was much less than in animals scalded to the head.[3]

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TABLE II. EFFECT ON LOCAL FLUID LOSS AND BLEEDING VOLUME OF DRUGS ADMINISTERED THIRTY MINUTES BEFORE THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)				BLEEDING VOLUME (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P*† VALUE	NUMBER OF MICE	MEAN	S.E.*	P*† VALUE
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	24	3.52	0.19	< 0.01	24	3.79	0.12	<0.01
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	24	3.99	0.21	< 0.01	24	3.90	0.10	<0.01
C	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	24	3.71	0.17	< 0.01	24	3.63	0.14	<0.05
D	Saline	0.2 c.c.	48	5.02	0.15		48	3.48	0.07	
E	No burn		12	0.39	0.07	< 0.01	37	5.63	0.07	<0.01

*Symbols and method of calculation same as Table I.
†In each case comparison is made with Experiment D.

TABLE III. EFFECT ON LOCAL FLUID LOSS OF DRUGS ADMINISTERED FROM FIVE TO EIGHT MINUTES AFTER THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P*† VALUE
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	24	4.07	0.17	> 0.4
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	24	3.90	0.17	> 0.1
C	Histamine diphosphate	0.1 mg. base in 0.2 c.c.	24	3.79	0.18	> 0.05
D	Saline	0.2 c.c.	24	4.24	0.19	

*Symbols and method of calculation same as Table I.
†In each case comparison is made with Experiment D.

TABLE IV. EFFECT ON ABSORPTION OF EDEMA FLUID OF DRUGS ADMINISTERED SIXTEEN HOURS AFTER THERMAL INJURY

EXPERIMENT	DRUG	AMOUNT INJECTED	LOCAL FLUID LOSS (AS PER CENT OF BODY WEIGHT)			
			NUMBER OF MICE	MEAN	S.E.*	P*† VALUE
A	Sodium pentobarbital	1 mg. in 0.2 c.c.	15	3.29	0.22	> 0.7
B	Morphine sulfate	0.5 mg. in 0.2 c.c.	15	2.98	0.18	> 0.1
C	Saline	0.2 c.c.	15	3.23	0.21	

*Symbols and method of calculation same as Table I.
†In each case comparison is made with Experiment C.

Effect on Local Fluid Loss of Drugs Administered Immediately After Burning: The same drugs were used as in the previous experiment (Table II, Experiment A), but they were given from five to eight minutes after scalding a single hind limb at 65° C. for ten seconds. The mice were bisected approximately four hours after trauma. As shown in Table III, each of these compounds failed to inhibit local edema formation.

Effect of Drugs on Absorption of Edema Fluid: As shown in the two previous experiments (Table II, Experiments A and B), sodium pentobarbital and morphine sulfate curtailed local edema formation when they were administered before, but not after, the burn. The following experiment was performed to

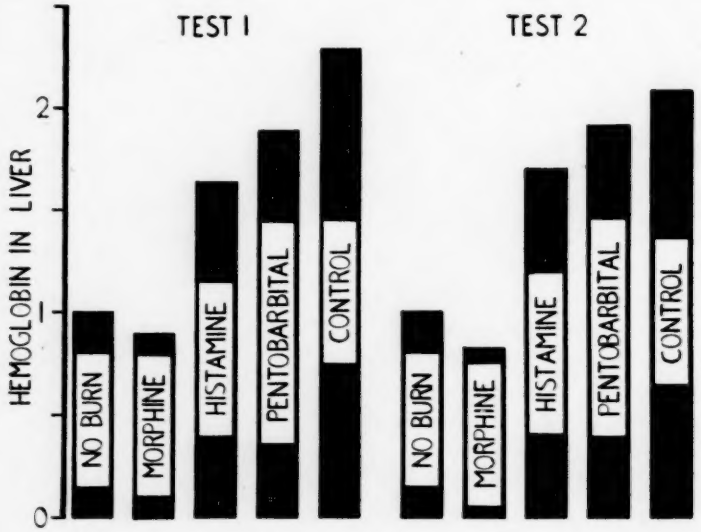

Fig. 1.—Effect of various drugs on hemoglobin retained in the liver. Morphine sulfate (0.5 mg. in 0.2 c.c. saline), histamine diphosphate (0.1 mg. base in 0.2 c.c. saline), sodium pentobarbital (1 mg. in 0.2 c.c. saline), and 0.2 c.c. saline (control), respectively, were injected intraperitoneally into mice (six mice per group). Thirty minutes after injection, both hind limbs of each mouse were scalded at 100° C. for twenty seconds, and the animals were exsanguinated three hours later. The amount of hemoglobin retained in the liver of each group of burned mice was compared with that in a parallel group of unburned animals.

ascertain whether or not either of these drugs would influence the absorption of fluid once edema had developed in the traumatized limb. One hind limb of three groups of fifteen mice each was burned at 65° C. for ten seconds. After sixteen hours, mice in two of the groups received an intraperitoneal injection of sodium pentobarbital and morphine sulfate, respectively, and those in the third group physiologic salt solution only. The dosage of each of the drugs was the same as in the previous experiments. From three to five hours after injection, the animals were sacrificed, and the amount of edema was determined by the bisection technique. As shown in Table IV, neither sodium pentobarbital nor morphine sulfate had any effect on the resorption of edema fluid.

Effect of Drugs on Capillary Atony.—In addition to local fluid loss, a second factor, that of capillary atony, is believed to contribute to the initiation of

burn shock.[2] This factor operates by causing stagnation of blood in atonic visceral capillaries, with a consequent reduction of the circulating blood volume.

In order to study the effect of sodium pentobarbital, morphine, and histamine upon capillary atony, the following experiments were performed. In each of two tests, five groups of six mice each were used. In four of the groups in each test, both hind legs of each animal were scalded at 100° C. for twenty seconds. Sodium pentobarbital, morphine sulfate, histamine, and physiologic salt solution, respectively, were injected intraperitoneally thirty minutes prior to the burn. The animals in the fifth group in each test were not subjected to a burn. The mice were exsanguinated at the end of three hours and the livers removed for estimation of hemoglobin. The amount of hemoglobin retention in the exsanguinated livers was used as an index of the degree of visceral congestion due to capillary atony. The method of estimating hemoglobin in the liver was described in the companion report.[1]

As shown in Fig. 1, morphine reduced the amount of hemoglobin retained in the liver of burned mice. The effect of pentobarbital and histamine upon the hemoglobin retention was not significant.

DISCUSSION

After it was shown that ethanol, prophylactically administered, increased survival time and diminished mortality in mice subjected to a standardized thermal injury,[1] other compounds related either chemically or pharmacologically were investigated for antishock action. Morphine and sodium pentobarbital (drugs having narcotic properties), histamine (a vasodilator), acetone (related chemically to ethanol), glycerol, and propylene glycol (alcohols) were all found to increase survival time and the number of survivors.

The mechanism of the antishock action of morphine, sodium pentobarbital, and histamine was investigated by observing their effect upon two major factors which are implicated in the initiation of burn shock, namely, local fluid loss and visceral congestion due to capillary atony.

The effect of these compounds upon edema formation in a burned limb was investigated. Each of these drugs curtailed the amount of fluid loss in the burned extremity, but only if administered before the thermal injury. None of these drugs had any effect upon the development of edema when injected immediately after the burn, nor did these agents hasten absorption of edema fluid when given after swelling of the scalded limb was well established.

Animals in which a single hind limb was burned exhibited a small, but significant, reduction in the circulatory blood volume as represented by the bleeding volume, and each of these drugs curtailed to some extent the expected reduction due to thermal injury.

A number of observers have noted antishock activity for barbiturates in animals subjected to manipulation of the intestines, to burning of the intestines, or to scalding of the skin.[6-10] Although Beecher and McCarrell[8] report sodium pentobarbital to be effective when injected after a burn in rabbits, we found this barbiturate, as well as morphine, histamine, and ethanol active only if given prior to the trauma. Beecher and co-workers[7, 8] attributed the thera-

peutic effect of the barbiturate to a curtailment of fluid loss from the burned surface. To explain this result, these authors suggested two possibilities; the one, reduction by the barbiturate of the flow of capillary filtrate due to capillary contraction and the other, lowering of the capillary pressure in the burned tissue. It is unlikely that the first of these possibilities holds for histamine or ethanol, inasmuch as these substances are known to dilate capillaries. Before the second explanation can be accepted or disproved, direct measurements of capillary pressure would be required. Another possibility that suggests itself is that barbiturates and the other drugs studied may have a specific action in decreasing capillary permeability at the site of injury. There is no clear-cut evidence for this, although Polderman and Beecher[11] have observed that the flow of cervical lymph is less under barbiturates than under ether. In summary the mechanism whereby these drugs prevent local fluid loss has not been satisfactorily explained.

The influence of each of these three substances upon visceral congestion due to capillary atony was investigated by estimating the amount of blood (hemoglobin) retained in the liver of exsanguinated mice. In burned animals which had received a prophylactic injection of moprhine, less hemoglobin was retained in the liver than in untreated, burned controls. Sodium pentobarbital and histamine were without significant effect. Previously, Castleman, who examined the tissue in the experiments performed by Beecher and McCarrell,[8] had noted that barbiturate diminished the amount of capillary congestion in the duodenal mucosa of rabbits with experimental skin burns.

It has been suggested by some investigators that histamine or related substances may be implicated in the production of the shock state.[12-14] In the present study, histamine was injected in amounts capable of producing some of the circulatory phenomena of shock;[15] yet an inhibitory action was exerted upon the expected reduction of the circulatory blood volume and the rate of local edema formation in burns. This would indicate that, in so far as burn shock is concerned, histamine is probably not the toxic factor involved in the production of the shock syndrome.

From the experiments which form the subject of this communication, it was concluded that morphine, sodium pentobarbital, and histamine owe their antishock action in burns, at least in part, to their inhibitory effect upon edema formation at the site of the burn, and that morphine in addition counteracts visceral congestion due to capillary atony.

SUMMARY AND CONCLUSIONS

Sodium pentobarbital, morphine, histamine, acetone, glycerol, and propylene glycol display antishock activity similar to that of ethanol. These substances increase survival time and diminish mortality in mice subjected to a standardized thermal injury.

The mechanism whereby morphine, pentobarbital, and histamine counteract shock was investigated by observing the effect upon two major factors implicated in burn shock, namely, local fluid loss and capillary atony.

Each of these drugs curtails edema formation at the site of the burn, but only if injected before the trauma. They neither prevent the development of edema nor accelerate the absorption of fluid when given after the burn. Morphine in addition counteracts visceral congestion due to capillary atony.

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THERAPEUTIC INDUCTION OF FEVER AND LEUCOCYTOSIS USING A PURIFIED TYPHOID PYROGEN

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A PURIFIED somatic antigen isolated from cultures of *Eberthella typhosa* grown on a synthetic medium was found to produce, in addition to the immunologic response, a febrile reaction followed by a secondary leucocytosis when injected intravenously in rabbits[1, 2] and man.[3] These properties of the antigen suggested its clinical application as a pyrogen in the induction of fever for therapeutic purposes in place of the whole typhoid-paratyphoid vaccine now generally employed.

MATERIALS AND METHODS

The original preparation of purified somatic typhoid antigen[2] had been stored in the refrigerator at 4° C. for from one to five years before use in these experiments. It consisted of a distilled water suspension containing 5 mg. of the antigen per cubic centimeter. The antigen, diluted in saline, was administered intravenously into the median basilic vein. Rectal temperature determinations were made at hourly intervals, and leucocyte counts were made at frequent intervals during the febrile paroxysms. Thirteen individuals, eight men and five women, eleven of whom had neurosyphilis, were selected for the induction of therapeutic fever.

RESULTS

A summary of the results obtained in six patients representative of the series is presented in Table I. The temperature and leucocytic response of a typical paroxysm are reproduced in Fig. 1. Following an adequate dose of the pyrogen, the temperature began to rise within from one-half to one hour after the injection and reached its fastigium in two or three hours, falling again to normal usually within from seven to eight hours. In Patients 2 and 4, the initial dose was small, since the toxicity of the preparation for man was not known. As a safety precaution the increments in the amount of antigen added in each succeeding dose were small. In subsequent cases an initial dose of 0.001 mg. was found to be safe. At first, the dose was usually doubled on each consecutive injection. It was soon found that this amount was not sufficient to obtain a consistently adequate febrile response. When a three- to fivefold increment in dosage was used, the results were usually satisfactory, as demonstrated with Patients 5, 7, 12, and 13.

With an adequate dose, the rectal temperature remained elevated at 103° F. or higher for from two to twelve hours, depending on the amount of pyrogen

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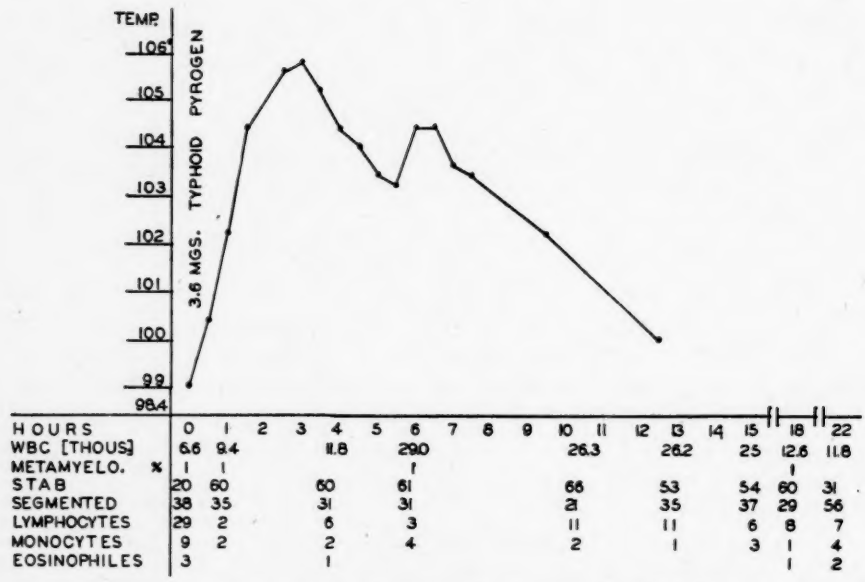
TABLE I. FEBRILE AND LEUCOCYTIC RESPONSE FOLLOWING INJECTION OF TYPHOID PYROGEN

PATIENT	DAYS AFTER INJECTION	AMOUNT OF PYROGEN (MG.)	MAXIMUM TEMPERATURE RECTALLY (° F.)	FEVER OF 103° F. OR MORE	LEUCOCYTE COUNT	COUNTED AFTER INJECTION (HOURS)
2	0	0.0001	100.1	0	–	–
	1	0.0002	101.8	0	9,000	3
	2	0.0003	101.2	0	–	–
	3	0.001	101.2	0	11,000	5
	6	0.002	101.6	0	–	–
	7	0.004	101.8	0	–	–
	8	0.01	104.4	0	7,000	5
	10	0.01	102.4	3	11,000	6
	11	0.01	102.0	0	8,000	7
	13	0.01	102.4	0	11,000	10
	14	0.01	101.2	0	8,500	5
	15	0.05	102.2	0	9,500	9
	16	0.10	102.6	0	9,500	5
	17	0.20	104.8	5	10,000	7
	22	0.20	104.0	2	17,000	10
	23	0.20	103.6	2	14,500	8
4	0	0.0001	101.6	0	10,000	5.5
	1	0.0002	102.0	0	9,000	5.5
	2	0.0005	101.6	0	9,000	5.5
	3	0.0025	104.6	4	–	–
	5	0.01	104.6	4	7,000	5.5
	7	0.05	105.0	5	–	–
	9	0.04	103.4	3	11,000	6
	11	0.1	105.2	5	28,000	9.5
	13	0.1	103.4	2.5	17,000	5.5
	15	0.3	105.6	5	11,000	5
	17	0.30	104.6	6	11,000	5
5	0	0.0002	100.2	0	9,500	6
	2	0.001	103.0	1	10,000	5
	4	0.007	105.0	2.5	22,000	13.5
	6	0.01	103.4	2.5	15,000	13.0
	9	0.05	102.6	0	10,500	6.0
7	0	0.0002	99.4	0	10,000	10.5
	1	0.002	101.0	0	17,000	5
	2	0.02	105.0	4	22,000	11
	5	0.02	103.0	2	29,000	12
	7	0.1	102.4	0	19,500	10
	13	0.5	105.0	3	37,500	14
	15	0.5	101.6	0	35,500	10
12	0	0.001	103.0	1	–	–
	1	0.005	103.8	3	–	–
	2	0.01	103.0	1	–	–
	3	0.05	105.0	6	–	–
	5	0.15	104.8	5	–	–
	7	0.6	105.0	8.5	–	–
	9	1.8	104.0	8.5	42,500	9
	12	7.2	105.4	9	33,700	9
	14	5.7	105.4	8	–	–
13	0	0.0005	99.8	0	–	–
	1	0.002	105.2	5	–	–
	2	0.005	103.8	3.5	–	–
	3	0.025	104.6	6	–	–
	5	0.1	105.8	4	–	–
	7	0.3	105.0	6	–	–
	9	0.9	104.6	5.5	31,800	9
	12	3.6	105.8	7	29,000	6
	14	14.4	105.4	12	–	–



HOURS	0	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	18	22
WBC [THOUS]	6.6	9.4			11.8		29.0				26.3			26.2		25	12.6	11.8
METAMYELO. %	1	1					1										1	
STAB	20	60			60		61				66			53		54	60	31
SEGMENTED	38	35			31		31				21			35		37	29	56
LYMPHOCYTES	29	2			6		3				11			11		6	8	7
MONOCYTES	9	2			2		4				2			1		3	1	4
EOSINOPHILES	3				1												1	2

Fig. 1 (Patient 13).—Typical febrile and leucocytic response following injection of typhoid pyrogen.

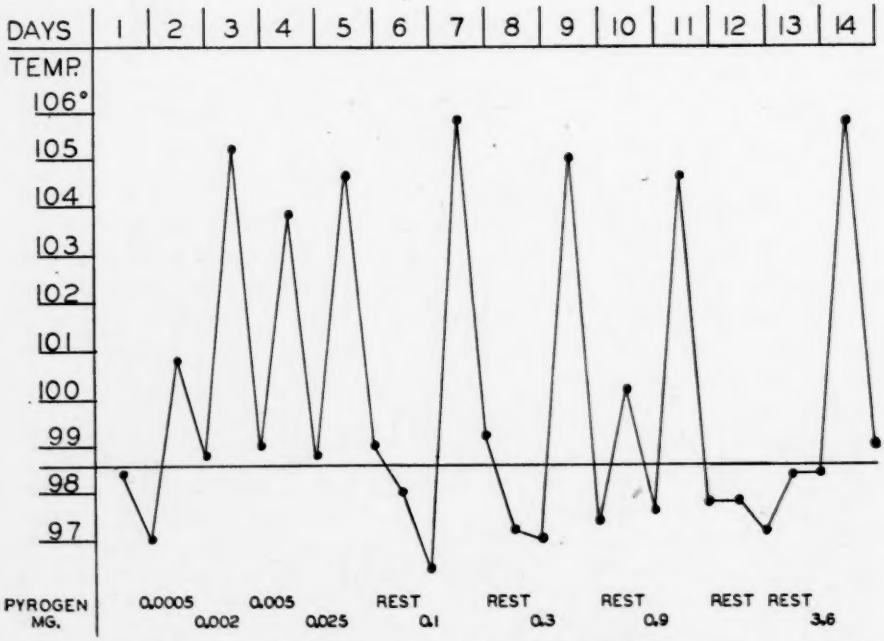


Fig. 2 (Patient 13).—Fever curve obtained with typhoid pyrogen.

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given and its relation to the dosage employed in the previous injection. When a fivefold or greater increase in dosage was used, the temperature rose to 104° F. or higher in most instances. However, as the amount of pyrogen given in a single dose grew larger (Patients 12 and 13), a temperature of 104° F. or more could be obtained by only tripling or quadrupling the preceding dose. These data show that the patients developed a tolerance for the fever-producing action of the substance.

Fig. 2 represents a typical course of febrile paroxysms produced in Patient 13. The lowest and the highest temperature of each paroxysm are recorded.

The leucocytosis developed within from three to five hours after the injection of the pyrogen and continued to increase for as long as fourteen hours. The total count often reached values of from 20,000 to 30,000 per cubic millimeter and occasionally higher, as shown in Fig. 1 and Table I. The differential counts showed that this elevation was due to an increase in the granulocytes, a large number being stab forms. The total leucocyte count often remained elevated for twenty-four hours or longer, particularly when larger doses of the pyrogen were used.

The systemic symptoms accompanying the administration of an effective dose of the antigen were quite uniform. Within from thirty to forty minutes after the injection, the patient began to feel chilly and developed a rigor of varying intensity which lasted for from twenty-five to thirty-five minutes. This was followed by generalized aching sensations which at times were accompanied by localized pain in the head, legs, back, and other parts of the body. The patient perspired during the height of the fever. Nausea and occasional vomiting also occurred. With the subsidence of the symptoms, there followed a feeling of fatigue and exhaustion. Only slight to moderate variations[3] in the blood values of glucose, chlorides, and carbon dioxide combining power appeared during the paroxysms, indicating that dehydration was not a factor in the development of the fever and leucocytosis. The ingestion of fluids during febrile periods probably aided in keeping the variations at a minimum.

DISCUSSION

A purified pyrogenic antigen isolated from cultures of *E. typhosa* grown in a synthetic medium has been shown to be an efficient, reliable substance for the induction of therapeutic fever. When an effective dosage has been established by preliminary testing, a series of febrile reactions of desired intensity can be induced with a single injection by increasing each consecutive dose by three- to fivefold as indicated in the results obtained in Patients 12 and 13.

The stability of the pyrogenic antigen makes it possible to use the standardized material for long periods of time. Patients 12 and 13 were treated with the same lot of pyrogen five years after Patients 2, 4, 5, and 7, with comparable results. During the five-year interval, the pyrogenic material was usually stored in the refrigerator at 4° C. but was kept at room temperature for as long as four days on several occasions. These results suggest that a large stock of the pyrogenic antigen can be prepared, standardized, and stored for long periods of time as needed for use in the induction of fever.

The stability and uniformity of the pyrogen and its effectiveness in small single doses make it preferable to the whole bacterial typhoid-paratyphoid vaccine now employed for the same purpose, which varies in its febrile inducing properties and which often requires multiple injections to obtain a single satisfactory fever response.[4] In addition, different lots of the bacterial vaccine have variable potencies in the induction of fever.

The marked leucocytosis which follows the injection of the pyrogen is probably a valuable adjunct in the therapeutic use of the substance.

SUMMARY

1. A pyrogenic somatic antigen has been isolated from cultures of *E. typhosa* which induces a febrile response in man when administered intravenously. A fever of from 103 to 105° F. follows doses of from 0.001 to 0.002 mg. To obtain similar results with subsequent injections, the amount of the pyrogen must be increased by three- to fivefold.

2. A granulocytic leucocytosis, with large numbers of nonfilament type of cells, accompanies and follows the febrile response.

3. When a given preparation of the pyrogen is standardized and stored in the refrigerator, it retains its potency for at least five years.

The authors wish to thank Dr. J. Gambescia and Dr. M. S. Ostrum, for their valuable assistance.

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COLORIMETRIC DIAGNOSTIC TEST FOR MALARIA

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INTRODUCTION

IN CHRONIC malaria and malaria in which parasites are not observed in stained blood films, it is important to have another means to aid in the diagnosis of the disease.

In 1928 Henry[1] described serologic tests based on the observation that sera from patients with malaria flocculate metharsenate of iron and melanotic pigment. The reaction containing iron was designated ferroflocculation and that which contained melanin and proved to be more sensitive, the melanoflocculation reaction. Grieg, van Rooyen, and Hendry[2] later made a complete study of the test. These investigators found the following disadvantages in the use of melanin prepared from ox choroid: (1) reactions from other protein substances, (2) inability to achieve complete uniformity in preparation of different batches of antigen, and (3) precipitation of stock choroid extract on storage. They prepared a melanin solution by hydrolysis of human hair, and the final extraction gave a solution which contained a fixed amount of pigment and remained stable. With this melanin extract they were able to change the technique of the test by discontinuing the preliminary titrations, increasing the sensitivity of the test, and using direct readings rather than electrometric devices. Even with this improvement the test was never used extensively, due to many false positive reactions.

Pewny,[3] in 1918, and Zieman,[4] in 1924, first attempted to demonstrate a precipitin reaction in malarial infections with limited success. In 1927 Taliaferro and co-workers[5] reported success with this type of reaction by using an antigen prepared from human parasites obtained from placentas. However, in a second report[6] they were not able to confirm their former findings fully. Row[7] later improved the test by preparing the antigen from malarial culture tubes of *Plasmodium vivax*. He also simplified the technique to obtain a strong uniform constant precipitin reaction in his hands. The difficulty in the preparation of sufficient quantities of antigen and the tendency of the test to give false negative reactions made this method impractical.

Coggeshall and Eaton[8] reported another diagnostic aid, the complement fixation reaction for chronic and apparently submicroscopic malaria. Their original work was done with monkey malaria, but later they were able to show similar results on human malaria[9] using the same antigen prepared from the monkey parasite *Plasmodium knowlesi*. Although specific in nature, the test is

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not refined sufficiently to detect all known positive cases. *Plasmodium gallinaceum* appears to be a fairly successful parasite for antigen in complement fixation but does not produce as sensitive an antigen as *P. knowlesi*, either extracted or used whole. Group studies in this laboratory, using *P. gallinaceum* antigen on known patients with malaria, gave approximately 35 per cent positive reactions. As yet the human malarial parasite has not been cultured,[9] so that sufficient quantities are available for a specific antigen.

All serologic reactions are basically the combination between antigen and antibody. The antigen in each case is usually specific, exceptions being those in the precipitation and complement fixation techniques in the detection of syphilis, in which some extract of beef heart is used, sheep cells in the heterophile reaction for infectious mononucleosis, etc. These serologic reactions are based primarily upon the coagulation and precipitation of a lyophobic colloidal dispersion in the presence of the serum containing specific antibodies or in some instances in the presence of a substance enhancing the differentiation of positive and negative sera.

Any type of test, either serologic or chemical, should be sufficiently sensitive to detect the weak positive or slight changes in the sera and should be specific for the disease without producing false positives in sera of patients with other diseases or normal individuals. The afore-mentioned tests do not meet these requirements, and it is apparent that an improved serologic or chemical test should be found which would enable one to follow the disease throughout its course.

The object of the present study was to develop a test, serologic or chemical, which would aid in the diagnosis of malaria. Much preliminary work was done in purifying *P. gallinaceum* antigen in attempts to improve the sensitivity and specificity, but it was found that purification did not increase the number of positive reactions in known positive sera, whether they were used in precipitation or complement fixation techniques. Melanin, used in previously mentioned tests,[1] presents in colloidal solution properties similar to those of solutions of certain colloidal dyes. Since colloidal dyes have been shown to combine quantitatively with proteins[10, 11] under certain conditions, and since they also provide a convenient basis for a colorimetric evaluation of the altered dye solutions, it was felt that the modified characteristics of malaria serum occurring during the acute and chronic phases of the disease might be demonstrated by the use of such dyes.

GENERAL METHODS

In the earliest tests using dyes (Evans blue T1824, atabrine, methylene blue, neutral red, brilliant cresyl blue, and Congo red), concentrations of from 0.1 to 1.0 per cent were tried in varying amounts with known positive and negative malarial sera. Although promising, no consistent results were obtained in these preliminary titrations. It was thought that electrolytes might enhance the activity of the dye, and further titrations were carried out using these substances. Varying concentrations of NaCl were tried, and in some of the titrations it was possible to increase the activity of the dye; however, the action did not appear to be constant.

Of the several dyes (indicators) and electrolytes* (enhancers) used, Congo red, a colloidal dye, and quinine HCl† gave the most constant and consistent results in the presence of malarial and nonmalarial sera. The following report contains data based on the use of a colorimetric test involving Congo red, quinine HCl, and malarial serum.

APPARATUS

The following equipment and chemically clean glassware are suggested for the most effective performance of the test. Proper and careful dilution is also essential for obtaining the best results.

Test Tubes.—

1. 13 by 100 mm.
2. 25 by 100 mm.
3. Graduated cylinders, 100 c.c.
4. Pipettes
   - 1 c.c. to 0.01 c.c. graduation
   - 10 c.c. to 0.1 c.c. graduation
5. Spectrophotometer‡
6. Cuvettes, 19 by 105 mm.

Chemicals.—

1. Congo red§
2. Quinine HCl, 0.3 per cent aqueous sol

Sera.—Cell-free, nonhemolyzed, nonchylous, and noninactivated.

PROCEDURE

One cubic centimeter of a 0.1 per cent solution of aqueous NaR (Congo red) is placed in a test tube (13 by 100 mm.), and 0.5 c.c. of the noninactivated serum to be tested is added. This mixture is vigorously agitated. Two cubic centimeters of quinine hydrochloride are then added to the Congo red serum mixture, and complete mixing is obtained by inversion of the test tubes. The rack containing the tubes is allowed to remain at room temperature for forty-five minutes. The tubes are centrifuged for ten minutes at from 3,500 to 4,000 r.p.m. One cubic centimeter of the clear supernatant is transferred to a graduated cylinder and diluted to 60 c.c. with distilled water. One drop of N/10 NaOH is added to the diluted mixture. A 19 by 105 mm. cuvette‖ is filled with distilled water as a reference standard, and the galvanometer needle is set to zero optical density on the scale with water in place in the cuvette well, using a wave length of 500 mm., the point of maximum light adsorption by the red-orange solution.

The optical density of each test solution is read in turn, and the values are recorded as being directly proportional to the dye left in solution.

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*Sodium chloride, N/100 HCl, quinine hydrochloride, ammonium sulfate, magnesium sulfate, caffeine sulfate, morphine sulfate, quinine sulfate, quinine bisulfate, and sodium sulfate.

†One of us (M. G. M.) suggested this compound, as it was felt that there might be a direct relationship of the compound and the malarial sera.

‡Junior spectrophotometer, model 6, Coleman Electric Co.

§National Aniline Division, Allied Chemical & Dye Corporation, New York, N. Y.; certified, 0.1 per cent aqueous sol.

‖Coleman Electric Co.

It was found that an aqueous solution of Congo red* with a pH of approximately 6.5 gave an optical density reading of 0.390 at a concentration of 1:250,000 and a wave length of 500 mm. The Congo red concentration in the test was chosen so as to give an optical density reading of approximately 0.400 with normal sera. The actual final dye concentration, provided none was precipitated, would be 1:210,000. Such a solution theoretically should possess an optical density of 0.465. This figure has yet to be attained. A dye-protein-quinine complex precipitate has always been obtained in small amounts, even in nonmalarial serum.

Tenth normal NaOH was found to be necessary to clear the final dilution mixture of a slight opacity which was encountered in some sera. One drop of the hydroxide is added to all tubes, whether opacity is present or not, to keep this added factor of dilution constant. Acid was tried as a clarifying agent but was found to modify the color of the remaining NaR sufficiently to give false and inconstant optical density.

The presence of a very chylous sera was found to have a direct bearing on the optical density. It is suggested in these cases that fasting sera be used in performance of the test.

When serum was inactivated at 56° C. for from fifteen to thirty minutes, the samples were always found to approach normal readings. Duplicate samples of sera from acute cases, inactivated and noninactivated, were tested. The noninactivated sera gave significantly low optical density readings, while inactivated sera were found to give no variation from the normal curve, whether the sera were from patients with or without malaria.

EXPERIMENTAL

Monkey Malaria.—Since the preliminary work showed optical differences between malarial and nonmalarial sera using the previously described dye-protein test, it was felt that this test could be used to follow the clinical course of the disease. An experiment with *P. knowlesi* infected *Macacus mulatta* monkeys was set up in an attempt to correlate the dye-protein results before, during, and after drug administration with simultaneous blood film examinations. Control readings on normal sera were taken on all monkeys for several days before infection, and on three of the monkeys seven readings were obtained over a period of nine days. Six monkeys were infected by intravenous inoculation of heavily parasitized *P. knowlesi* blood. The course of the disease was followed by daily thick and thin smears, and dye-protein determinations were made on sera at varying intervals. Smears were taken from peripheral blood, while from 3 to 4 c.c. of blood were drawn from the femoral vein for the dye-protein test.†

Results: The results of the dye-protein tests on serum from monkeys infected with *P. knowlesi* are shown in Fig. 1.

*As each lot of dye may vary, it is recommended that concentration-optical density curves be set up in each laboratory and that these data be used to select wave length and dilution ratios.

†Dye-protein reaction time increased to sixty minutes and centrifugation speed to 4,500 r.p.m. when monkey sera was tested.

Optical density readings in Monkey 0 before infection varied from 0.400 to 0.460 in eight serum samples taken over a ten-day period before infection. Three days after infection the optical density reading was observed to be 0.420, but readings fell steadily thereafter to 0.190 on the sixteenth day of infection. This animal was then treated with atabrine HCl. The readings of serum samples tested during the next six days gradually approached normal and remained there in subsequent samples.

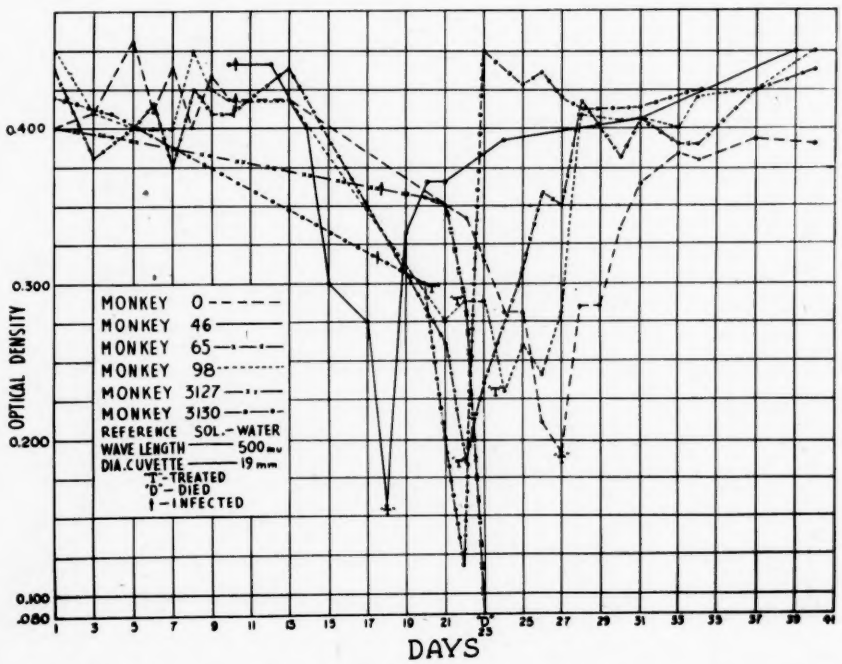

Fig. 1.—Optical density curves of dye-treated sera from monkey infected with *Plasmodium knowlesi*.

Preinfection normal optical density readings of serum samples from Monkeys 46, 65, and 98 varied from 0.380 to 0.450. Only one sample was taken before inoculation of the parasite into Monkey 46. Another serum sample taken two days later from this animal was found to give a similar reading, 0.440. The readings fell gradually during the course of the disease, reaching 0.160 in Monkey 46 on the eighth day of infection and 0.185 in Monkey 65 and 0.285 in Monkey 98 on the eleventh day of infection. After treatment the optical density readings in Monkeys 46 and 65 rapidly approached the normal range within four days, while serum from Monkey 98 dropped to 0.230 two days after treatment was instituted. Four days after further treatment serum samples from this monkey gave normal readings.

Monkey 3127 had the same normal optical density readings as shown for the previously described monkeys but failed to respond to treatment and died. The serum taken immediately before death gave a reading of 0.080.

Chicken Malaria.—The results of the dye-protein test using sera from monkeys suggested further trial using a different parasite and host. Chickens furnished the most economical and convenient host.

Four-week-old chicks were infected with *P. gallinaceum*. One-fourth cubic centimeter of heavily parasitized blood was given intravenously. Control blood levels were taken on all chickens before infection and also on a small group of noninfected birds to ascertain if daily bleedings of from 2 to 3 c.c. were detrimental to the animal or caused enough changes in the blood to change the test readings significantly.

A group of forty-three birds was used in the experiment. Thirty-seven birds were infected and six were used as normal controls. Blood was obtained by heart puncture. Treatment was instituted when the blood picture and gross physical condition of the individual chicken appeared to warrant it. Five-tenths cubic centimeter of a 3.0 per cent solution, quinine HCl, was given intramuscularly.

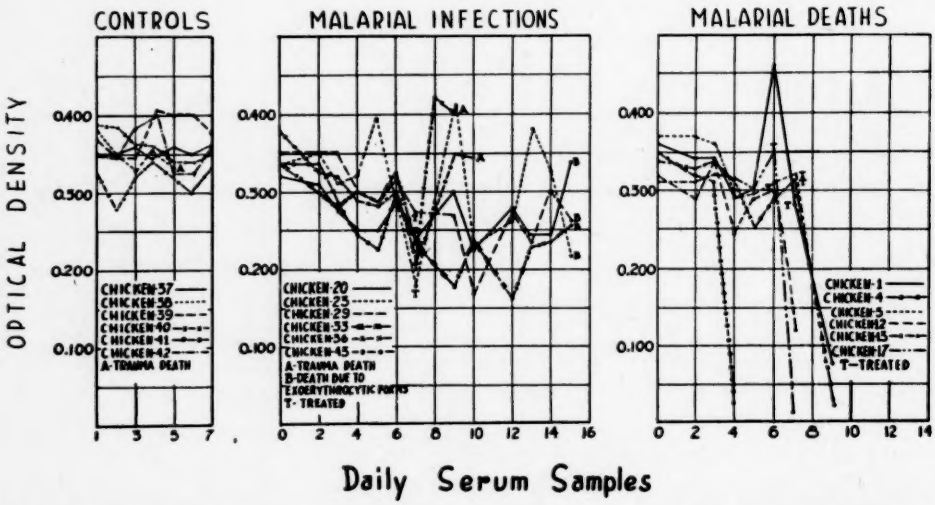

Fig. 2.—Optical density curves of dye-treated sera from chickens infected with *Plasmodium gallinaceum* and controls.

Results: The optical density readings of eighteen chickens are shown in Fig. 2.

In the six control noninfected chickens daily bleedings appeared to have no apparent effect on the blood serum. The normal readings varied between 0.280 and 0.405. One bird, Chicken 41, had consistent low readings varying between 0.280 and 0.340. These findings are depicted in the left side of Fig. 2.

Sixteen of thirty-seven infected chickens died of other causes than malaria. Fifteen of these sixteen chickens died of trauma due to heart puncture, while autopsy on the remaining bird showed pulmonary involvement.

The course of the disease with treatment as interpreted by the dye-protein test is shown in the central portion of Fig. 2. The normal optical density readings of the six chickens correlated with the normals obtained in the control group.

These chickens were infected shortly after the control samples of blood were taken. Four chickens were blood positive the second day after infection, with the remaining two, Chickens 20 and 29, becoming positive on the third day. At that time the readings were gradually falling. On the sixth day there was a decided upswing in the optical density curves of five of the chickens. This same phenomenon was observed in the sixth bird, Chicken 23, on the fifth day. The parasitized red cell percentages on these chickens at the time of this rise in the curves was found to be between 50 and 80. On the seventh day of infection the curves of the six chickens shown in this central graph were observed to fall dramatically, and at this time they were treated. Within twenty-four hours the curve of Chicken 36 rose from 0.210 to 0.420. The optical density curves of Chickens 23 and 43 showed a similar rise in forty-eight hours. The parasite percentages in the latter two were less than 5, and in Chicken 36 it had dropped to 40 from 70 the preceding day. Chickens 36 and 43 died of trauma on the ninth and tenth days. The curve of Chicken 20 was observed to rise from 0.240 to 0.300 forty-eight hours after treatment, falling again with a gradual rise until the day of death. The curve of Chicken 29 was observed to fall gradually until the tenth day at which time the optical density reading began to approach 0.300. Chicken 33 continued to have low readings until the twelfth day at which time there was an upswing toward the normal range. The parasite percentages of these chickens were less than 5 with the exception of Chicken 20 which had had approximately 50 per cent parasitemia throughout the course of the disease. On the fifteenth day of infection Chickens 20, 23, 29, and 33 died. Subsequent brain smears on these birds showed the presence of exoerythrocytic forms of the malaria parasite.

The right side of Fig. 2 depicts the optical density curves of chickens dying before or at the time treatment was instituted. The curves of the chickens in this group follow a similar pattern to those described in the central portion of Fig. 2. There is a general upswing in the curves with a dramatic drop in the next day or two, whether the chickens were treated or not. All birds in this group died of the blood phase of the disease.

Human (Vivax Malaria).—The shape of the optical density curves in the experimental disease in monkeys and chickens suggested that the dye-protein test would serve as a diagnostic aid in human malaria. It was also thought that response to treatment might be followed with the test.

A minimum optical density of 0.340 and a maximum of 0.460 with a mean of 0.390 has been found on twenty-five of nonmalarial human controls without malaria. As no attempt is being made at this time to report on a large series of patients, it is felt that the curves on the sera of seven patients with recurring vivax malaria will demonstrate the principle of the test. Several of the patients have been followed as long as from two to eight months.

Fig. 3 shows the dye-protein curves of human patients with malaria over a perior of fifteen weeks. The first blood drawn from the dye-protein test is taken during or shortly after a relapse. This procedure was followed in all patients.

The first optical density reading obtained on Patient 25 was 0.240 with the blood being drawn shortly after a relapse. Within one week the curve had approached 0.330 and remained approximately at that level until the fifth week. The curve gradually declined until the eighth week at which time the patient had another relapse, and a reading of 0.185 was obtained. Three weeks later the optical density curve had reached 0.350.

Patient 26 gave an original reading of 0.240 during a relapse. In two weeks the curve had reached 0.340 and remained above 0.350 throughout the period of thirteen weeks that this patient's serum was tested. During this period the patient had no relapses, and no parasites were observed on thick smears.

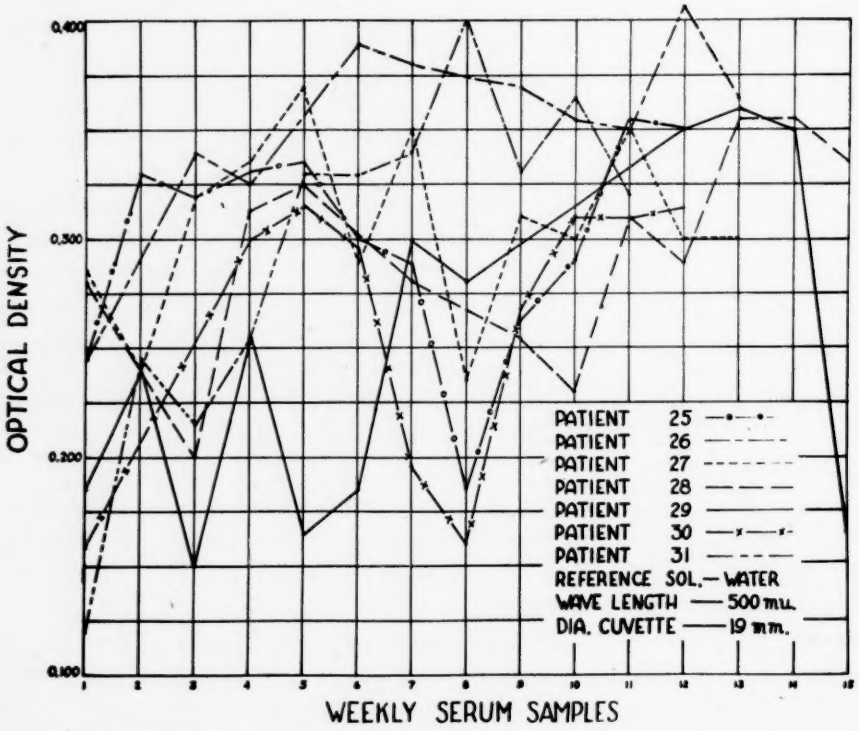

Fig. 3.—Optical density curves of dye-treated sera from human patients infected with *Plasmodium vivax*.

The original optical density reading and all others except the last recorded on the graph were made on Patient 27's serum at other times than during an acute relapse. Health records show eight relapses, while the patient states that he had twenty-nine relapses. A constant parasitemia was observed by blood smear. The readings obtained on serum from this patient over a thirteen-week period ranged between 0.300 and 0.350. Although this patient was in constant parasitemia, only a few parasites were found at each examination.

The original reading from Patient 28 was obtained during subjective symptoms. The optical density curve dropped to 0.200 two weeks later during a clinical relapse. The curve then gradually rose to 0.325 in the next two weeks but

started another gradual decline reaching a low point of 0.230 in the tenth week during a relapse. This patient's curve again rose to 0.350 in the next three weeks and remained there during the follow-up period. Blood smears were positive at the time of clinical relapses.

The first optical density reading of Patient 29 was 0.190 and was obtained during a relapse. This patient also had a constant parasitemia as shown by thick smear. After the original relapse as followed in this study, he relapsed three weeks later giving a reading of 0.150 from a 0.240 rise during the second week. Two weeks later there was another dramatic fall in the optical density curve to 0.165 from 0.255. From this low reading during the fifth week, there was a gradual rise reaching 0.360 in the thirteenth and fourteenth weeks. The following week this patient had another relapse with the curve dropping to 0.165. From the seventh to the fourteenth week the blood smears on this patient were negative, becoming positive at the time of the last recorded relapse on Fig. 3. This patient stated that he had had thirty relapses with an official record of only four in his health record.

Patients 30 and 31 showed very low optical density readings (0.165 and 0.090) during the first relapses to be recorded in this study. There was a gradual rise of both curves to 0.300 or greater with the readings on Patient 31 going as high as 0.400 without further relapses being observed during the study. Patient 30 relapsed in the eighth week with an accompanying lowered reading of 0.160. The optical density curve on this patient again rose to 0.300 and remained at this level during the study. Positive blood smears were observed during the relapse periods of these patients.

DISCUSSION

The present investigation brings into prominence severeal possibilities of explanation which cannot be further studied at the present time. It was noted, for example, that the quantity of serum used in the test apparently produces no effect upon the optical density readings providing the dye concentration remains unaltered. Variations in quinine hydrochloride concentration produce changes in the readings which are reproducible. Quinine hydrochloride alone will precipitate NaR from solution, while serum and NaR alone give no apparent reaction. At first it was thought that serum acted as a protective colloid in modifying the action of quinine hydrochloride on NaR, but the unchanged readings using variable amounts of sera seem to preclude this theory. It is possible that the A/G ratio is important in the reaction, especially since $(NH_4)_2SO_4$ precipitated globulin produced an effect similar to whole serum in the test, while $(NH_4)_2SO_4$ precipitated albumin showed no comparable change. It is also possible that the reaction is a combination of globulin denaturization by quinine hydrochloride, A/G ratio, and direct action of quinine hydrochloride and NaR. Somewhere in the interpretation of the test must be considered the drastic changes produced by inactivation of the sera at 56° C. Also, CO_2 frozen sera upon thawing gave optical density readings which did not correspond to results obtained with untreated sera. No correlation of this data has been attempted.

It is possible that the reaction herein described bears no specific relationship to a malarial infection but has as its basis the presence of certain denaturized proteins resulting from the response of the parasite or host, singly or in combination. However, controls other than serum from patients with infectious hepatitis or marked anemia showed no similar reactions.

Although the chemoimmunologic basis of the test is not as yet understood, it is believed that the principle and technique described could be elaborated upon and might possibly be utilized as a significant diagnostic aid in malaria. With the use of different dyes and enhancers, modifications of concentrations, and techniques or other possible changes, it might be possible to make this dye-protein reaction applicable to other diseases.

SUMMARY

A dye-protein test involving the use of Congo red, quinine hydrochloride, and serum has been described in which it is possible to detect serum changes in malarial infections. Observations on experimental and human infections lead one to believe that the test could be utilized as a diagnostic test for latent infections. The technique of the test is not presented as a definitive procedure but rather as one that has sufficient promise to warrant further exploration.

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HEBERDEN'S NODES

V. The Association of Hypertension and Obesity to Degenerative Joint Disease of the Fingers

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Cleveland, Ohio

HEBERDEN'S nodes are enlargements of the terminal joints of the fingers due to degenerative joint disease. Since numerous observers have noted that degenerative joint disease and hypertension have occurred together, the present study was planned to investigate the blood pressure of women with Heberden's nodes and to determine whether or not this particular form of degenerative joint disease was associated with high blood pressure.

Swett[1] noted the association of arterial hypertension and chronic arthritis. Archer[2] studying the menopause type of degenerative arthritis found that six of his twenty patients had blood pressure over 150 mm. Hg. Bick[3] in a study of 200 cases of chronic arthritis said that the blood pressure in osteoarthritis in most cases is considerably above normal for each age group. O'Reilly[4] found hypertension in twenty of fifty-six cases of osteoarthritis. Weber[5] found an average blood pressure of 142 mm. Hg in twenty-one cases of hypertrophic arthritis compared to 127 mm. in atrophic arthritis. Fletcher[6] in 103 cases of osteoarthritis found obesity, hypertension, and raised erythrocyte sedimentation rate as associated characteristics. He found that a significant proportion of his patients, 36 per cent of those of normal weight and 52 per cent of those with obesity, had hypertension. Most recent students of arthritis have ignored the question.

Heberden's nodes are a particular manifestation of degenerative joint disease which involves the terminal finger joints. Many writers have stressed the fact that Heberden's nodes are typical of degenerative joint disease and are the most common manifestation of this disease. Previous studies[7] indicate that Heberden's nodes occur frequently as the only significant evidence of degenerative joint disease, that heredity[8] is an important factor in their occurrence, and that their development depends also to a large degree upon age and sex.[9]

The present study is based on 112 women with idiopathic Heberden's nodes who were seen on the medical service at City Hospital or in private practice. The diagnosis of idiopathic Heberden's nodes as distinguished from traumatic Heberden's nodes depended upon enlargement and deformity of at least three fingers on both hands occurring without relation to trauma. After the patients of this series had been assembled, they were arranged in order of age and divided into groups by decades. Patients were considered to have an abnormally high blood pressure if they had a systolic pressure of

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TABLE I

HEBERDEN'S NODES SERIES 112 WOMEN								CONTROL SERIES 92 WOMEN							
AGE	NUMBER	AVERAGE AGE	AVERAGE B.P.	RANGE B.P.	STANDARD DEVIATION	NUMBER WITH HIGH B.P.	PER CENT WITH HIGH B.P.	AGE	NUMBER	AVERAGE AGE	AVERAGE B.P.	RANGE B.P.	STANDARD DEVIATION	NUMBER WITH HIGH B.P.	PER CENT WITH HIGH B.P.
30 to 39	3	37.0	126	120 to 140	± 9.4	0	0	30 to 39	10	35.0	115	100 to 130	±10.6	0	0
40 to 49	21	45.3	145	108 to 228	±26.4	7	33	40 to 49	26	44.5	143	90 to 210	±31.7	7	27
50 to 59	36	54.6	153	100 to 240	±49.8	16	41	50 to 59	26	54.0	154	108 to 210	±27.9	11	42
60 to 69	36	63.7	163	120 to 220	±27.4	22	60	60 to 69	18	64.4	157	115 to 218	±26.1	9	50
70 to 84	16	75.0	188	144 to 240	±26.8	14	88	70 to 84	12	73.0	163	122 to 205	±25.2	6	50
	112				±27.2	59	53		92				±27.02	33	36

over 159 mm. Hg. This designation was chosen regardless of diastolic pressure. Only five patients in the study series and three in the control series would have been added to the high blood pressure group if a diastolic pressure of 90 mm. Hg or over also were accepted.

The limit of 160 mm. Hg was chosen to be liberal because these women were not hospitalized or examined under basal conditions, and many of them were seen only once. Page and Corcoran,[10] quoting Symonds, give normal blood pressure in 150,419 healthy men as never higher than 135 mm. Hg. White[11] states that normal systolic pressure ranges from 95 to 145 mm. Hg. Bramwell[12] stresses the fact that there is a range of normal blood pressure and that 150 mm. Hg or less is normal. Robinson and Brucer[13] state that the normal range of blood pressure is from 90 to 120 mm. Hg systolic. Hines considered 160/100 mm. Hg the upper limit of normal.

The patients with Heberden's nodes are compared to a control series of ninety-two women chosen previously so as to simulate closely in age and sex distribution the original Heberden's node series studied for heredity.[14] As will be noted from Table I, the two series are quite similar in size and age distribution. The average blood pressures in each age group are nearly the same in both series, being only a trifle higher in the Heberden's nodes series. The range of blood pressures in each group is great. This, and the fact that the series are small, explains why the standard deviation is so great. These differences in blood pressures between the two groups are not statistically significant. Even in the eighth decade where the difference is greatest, the mean blood pressures in the two groups are 188 and 163, less than the standard difference of the series.

The percentage of women with high blood pressure may seem unduly high, but it does not vary widely from those found in the population in general. Master and Marks[15] found the percentage of women with hypertension (systolic pressure over 150 mm.) to be 20 in the forties, 43 in the fifties, 63 in the sixties, and 68 in the seventies. The Metropolitan Life Insurance Company[16] found that 20 per cent of women at 40 years of age had hypertension (systolic pressure over 140 and a diastolic pressure over 90).

Although there was no significant difference in the incidence of hypertension between the affected and the control group, the relationship of hypertension to Heberden's nodes was further tested by combining the two series into one, comparing the incidence of hypertension in the Heberden's nodes and the normal series, and computing the coefficient of association between Heberden's nodes and high blood pressure. This has been done in Table II. The coefficient of association was found to be 0.25.[17] According to the computation used an answer of +1 indicates that the attributes under consideration are invariably associated with each other, neither one occurring independently of the other. When the answer is −1 there is complete negative association and neither attribute ever occurs in association with the other. The result found here, 0.25, must be interpreted as of no significance.

TABLE II. HEBERDEN'S NODES AND HYPERTENSION FOUND IN TWO HUNDRED AND FOURTEEN WOMEN

AGE	NUMBER	HEBERDEN'S NODES AND HYPERTENSION	HEBERDEN'S NODES WITHOUT HYPERTENSION	HYPERTENSION WITHOUT HEBERDEN'S NODES	NO HEBERDEN'S NODES NO HYPERTENSION
30 to 39	13	0	3	0	10
40 to 49	47	7	14	7	19
50 to 59	62	16	20	11	15
60 to 69	64	22	24	9	9
70 to 79	28	14	2	6	6
Total	214	59	63	33	59

TABLE III. HEBERDEN'S NODES FOUND IN EIGHTY-TWO WOMEN WITH HYPERTENSION

AGE	NUMBER	NUMBER AFFECTED	INCIDENCE FOR AGE	EXPECTED NUMBER AFFECTED
40 to 49	8	0	0.010	0.08
50 to 59	35	1	0.026	0.91
60 to 69	24	2	0.155	3.72
70 to 79	15	1	0.247	3.70
	82	4		8.41

TABLE IV. HEBERDEN'S NODES AND OBESITY IN ONE HUNDRED AND EIGHTY-FOUR WOMEN

AGE	NUMBER	HEBERDEN'S NODES AND OBESITY	HEBERDEN'S NODES WITHOUT OBESITY	OBESITY WITHOUT HEBERDEN'S NODES	NO HEBERDEN'S NODES NO OBESITY
30 to 39	13	1	2	3	7
40 to 49	40	6	8	11	15
50 to 59	50	7	17	12	14
60 to 69	53	11	24	5	13
70 to 79	28	4	12	1	11
Total	184	29	63	32	60

As a further test eighty-two women with hypertension were examined on the wards or in the clinic of City Hospital in a systematic search for Heberden's nodes in such a group. Table III shows that four were found, only one-half of normal expectation in a series of this size and age distribution. The expected number affected was compiled from the incidence actually found in a routine observation of nearly 7,000 individuals.[9] The series is small and too much reliance cannot be placed upon it, but the findings do not support the supposition that Heberden's nodes and high blood pressure are attributes associated with each other in a proportion higher than in the population at large.

The relationship between Heberden's nodes and obesity was then investigated. An association between obesity and degenerative joint disease has been recognized. O'Reilly[4] found that 12 per cent of his patients with degenerative joint disease were overweight. Weber[5] in an analysis of 150 patients noted that patients with degenerative joint disease had an average weight 16 pounds greater than patients with rheumatoid arthritis, despite the fact that they were 0.3 inch shorter. Fletcher[6] found that obesity occurred eight times as commonly in patients with degenerative joint disease as it did in the population in general. Kovacs and Hartung[18] found that fifty patients with degenerative joint disease weighed an average of 20 pounds more than fifty other

patients with rheumatoid arthritis. Haden and Warren,[19] studying fifty patients with degenerative joint disease, found that 62 per cent of the group weighed more than normal for their age, the average overweight being 25 pounds. Matz,[20] in 169 ex-service people with degenerative joint disease, found that 45 per cent were more than 5 per cent over standard weight. The women in our series were judged to be overweight if they were 20 per cent or more above the ideal weight for height and age as given in standard tables. Again the Heberden's nodes and control series were combined (Table IV). The coefficient of association of Heberden's nodes and obesity is 0.08 and therefore of no significance whatever.

COMMENT

The validity of this study depends upon the reliability of the observations and the selection of the material observed. The women with Heberden's nodes were selected solely because of the disease of their fingers in a study which took no account of blood pressures. This was random sampling so far as blood pressure was concerned. The control series is composed of the sisters of women who had no arthritis. They were chosen in the course of a previous study to compare the incidence of Heberden's nodes in the sisters of women without arthritis with the sisters of women with Heberden's nodes. The only other selection exercised was aimed to assemble a group with the same age distribution as the study series. No attention was devoted to blood pressure or obesity at the time other than recording these data. Eighteen of the women in the Heberden's nodes series were private patients. The other seventy-four women of the Heberden's nodes series, and all the members of the control series, were dispensary patients. The members of both series had lived in Cleveland and its environs all their lives. In general the people under consideration were in the modest or low income groups, many but not all of whom had done hard work. The two series seemed to be closely matched in all observable qualities except the presence of Heberden's nodes. According to the literature cited there seems to be an association of degenerative joint disease and hypertension. In no instance had the data presented been subjected to statistical analysis to determine the degree of significance of the findings. Furthermore, there was no comment as to whether or not the incidence of hypertension in patients with degenerative joint disease was any higher than that found in the population in general. Several of the studies cited[15, 16] indicate that it was not. After comparing the data from the literature with the findings of the study, it seems safe to conclude that patients with Heberden's nodes do not differ from those with degenerative joint disease of other joints in so far as the incidence of hypertension is concerned. No significant association exists between the two conditions.

Data from the literature indicates a strong association between degenerative joint disease and obesity. This association has been based upon very small deviations from standard weight, in some instances as small as 5 per cent in one study.[20] In two studies[5, 18] patients with degenerative arthritis were compared to patients with rheumatoid arthritis, a disease frequently

associated with debility and loss of weight. In four studies[4, 6, 19, 20] comparison was made to standard tables or to the incidence of obesity in the general population. In the present study no difference was observed in the incidence of obesity in Heberden's nodes and the control series. In respect to obesity, patients with degenerative joint disease of the fingers differ widely from patients with degenerative joint disease of other joints.

SUMMARY

A comparison was made between 112 women with Heberden's nodes and ninety-two women of approximately the same age distribution but otherwise selected at random. It was found that average blood pressures for each age decade were the same in each group. After combining the series into one group, no significant association between Heberden's nodes and hypertension could be demonstrated. In a series of eighty-two women with hypertension, Heberden's nodes were not found more frequently than could be expected from the incidence of this disease in the population in general. It is concluded that Heberden's nodes or degenerative joint disease of the fingers is no different, in relation to hypertension, than is degenerative joint disease of other joints. Despite the fact that a strong positive association has been demonstrated between degenerative joint disease and obesity, no such association was found in this series between Heberden's nodes and obesity. In this respect Heberden's nodes or degenerative joint disease of the fingers differs widely from degenerative joint disease of other joints.

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LABORATORY METHODS

THE DETERMINATION OF SULFONAMIDES IN TUNGSTIC ACID (FOLIN-WU) BLOOD FILTRATES

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SIMULTANEOUS determinations of blood urea, nonprotein nitrogen, sugar, etc., and the unconjugated sulfonamides are frequently required in research or are requested in the clinical laboratory. Hence a method for sulfonamides on routine, tunstic acid (Folin-Wu) blood filtrates should be helpful.

In the present paper are described colorimetric and photoelectric adaptations of the well-known Bratton and Marshall[1] procedures to tungstic acid blood filtrates. The conjugated sulfonamides were not investigated. While it was found necessary to study rather extensively many factors relating to these methods, only a few of the more important findings can be reported in detail.[2-4]

EXPERIMENTAL

Ordinary routine blood filtrates generally contain tunstate ions. Upon addition of the usual Bratton-Marshall reagents, these ions produce turbidities and also inhibit coupling of the diazotized sulfonamide and the color reagent. However, it was found that citric acid would inactivate these tungstic acid ions, if they are present in but small quantities.[5] The addition of either p-toluenesulfonic, trichloroacetic, or dilute sulfuric acid was also found necessary in order to stabilize and keep in solution the resultant dye. Under these conditions a deep, clear, rose-red or orange-red solution results.

Reagents.—

Sodium Nitrite: A 0.1 per cent solution is prepared weekly from a 10 per cent solution which seems relatively stable when kept in the cold.

Sulfamate Buffer Solution: Ammonium Sulfamate,* 0.5 Gm., and sodium acid phosphate ($NaH_2PO_4 \cdot H_2O$), 13.8 Gm., per 100 c.c. of solution.

Color Reagent: N-(1-naphthyl) ethylenediamine dihydrochloride,* 100 mg., are dissolved in 100 c.c. of water and preserved in a dark bottle.

Stabilizing Solutions: P-toluenesulfonic acid monohydrate,† 75 Gm. are dissolved in water and decolorized by boiling with Norit A‡ decolorizing carbon. The filtered solution is diluted to 500 c.c., and 10 Gm. crystalline citric acid are added. After the present work was partially completed, it was noted that the use of p-toluenesulfonic acid is rather expensive for routine hospital work. Two

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*LaMotte Chemical Products Co., Baltimore, Md.
†Eastman Kodak Company, Rochester, N. Y.
‡Pfanstiehl Chemical Co., Waukegan, Ill.

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per cent citric acid in N/5 H_2SO_4 was then found to constitute a satisfactory stabilizing solution for all sulfonamide determinations studied except sulfathiazole. The dye which sulfathiazole yields upon coupling with this color reagent under these conditions is insoluble, except in the presence of an acid such as p-toluenesulfonic acid or trichloroacetic acid. Hence, for sulfathiazole determinations, the best stabilizing solution found is a 20 per cent p-toluenesulfonic-2 per cent citric acid solution. These solutions are diluted 1 to 1 with water for the photoelectric method.

Standard Sulfonamide Solutions: Sulfanilamide, 1,000 mg., sulfapyridine, 250 mg., sulfamethazine, 250 mg., sulfathiazole, 250 mg., and sulfamerazine, 250 mg., are dissolved in N/11.4 H_2SO_4 and then made up to 1,000 c.c. with the acid. While water could be used for these standard solutions, the sulfonamides are more soluble in dilute acid. Furthermore, these solutions were also used in the recovery experiments to be described later in this paper. One hundred milligrams of sulfadiazine is made up to 2,000 c.c. in N/11.4 H_2SO_4. Appropriate working standards are prepared by dilution of accurately measured quantities of the stock solutions with distilled water. In routine work all standards need not be prepared. The values for the various colorimetric or photoelectric readings, except for sulfanilamide, can be obtained from a conversion table or standard calibration curve given by a comparative analysis of the various sulfonamides.

Sodium Tungstate Solution: A 10 per cent solution is carefully prepared by dissolving 100 Gm. in water and diluting to 1,000 c.c. This solution must not be too alkaline. Thus, 10 c.c. of the tungstate solution to which 5 c.c. of N/10 HCl has been added should require at least 4.7 c.c. of N/10 NaOH for neutralization to phenolphthalein.

PROCEDURES

Colorimetric Adaptation of the Bratton-Marshall Method to Tungstic Acid Filtrates.—One volume of blood is hemolyzed with eight volumes of N/11.4 H_2SO_4.* It is usually well to add an extra drop of N/1 H_2SO_4. If less than 5 c.c. of blood were collected in the oxalate bottle, however, a drop of 5 N H_2SO_4 must be used for hemolysis in addition to the usual amount of N/11.4 H_2SO_4. One must never neglect this precaution. This amount of acid lowers the pH of the filtrate to about 3 to 2.4 and minimizes the escape of tungstate ions. The decrease in pH does not seem to interfere with any of the routine blood chemistry determinations. The blood proteins are precipitated in the usual manner with 10 per cent sodium tungstate.

*In our hospital this slightly stronger acid is used, instead of the N/12 H_2SO_4, in order to correct for the alkalinity of the potassium oxalate used to prevent blood coagulation. One cubic centimeter portions of a 2 per cent potassium oxalate and 0.3 per cent sodium fluoride solution are evaporated to dryness in small bottles and used for the collection of routine bloods. This amount of anticoagulant is sufficient for from 15 to 20 c.c. of blood. Thus the concentration of oxalate will depend upon the amount of blood obtained from the patient. If small samples of blood (from 1 to 2 c.c.) are withdrawn, the increased concentration of potassium oxalate will neutralize some of the sulfuric acid and consequently will permit an excess quantity of tungstic acid ions to escape into the filtrate. Accordingly, after diazotization and addition of the color reagent, the solution will be turbid and will have a violet instead of a red color. Also, the dye will immediately flocculate. While in research work the correct relation of oxalate to blood (from 1 to 2 mg. per cubic centimeter) can be readily maintained, this is not practical in routine hospital work.

TABLE I. COMPARISON OF COLORS GIVEN BY VARIOUS SULFONAMIDES

SULFONAMIDE	MG. PER 5 C.C.	COLORIMETER READING (MM.) SET AT	COLOR	CONVERSION FACTOR (PERCENTAGE)
Sulfamerazine	0.1	20.0	Rose red	100.0
Sulfathiazole	0.1	18.5	Rose red (violet tinge)	108.1
Sulfamethazine	0.1	20.8	Rose red	96.15
Sulfapyridine	0.1	19.5	Rose red	102.6
Sulfadiazine	0.1	22.0	Red (orange tinge)	90.9
Sodium sulfadiazine	0.1	24.0	Red (orange tinge)	83.3
Sulfanilamide	0.1	16.7*	Orange red	119.8*
Sulfamerazine	0.05	20.0	Rose red	100.0
Sulfathiazole	0.05	18.8	Rose red (violet tinge)	106.4
Sulfamethazine	0.05	20.8	Rose red	96.15
Sulfapyridine	0.05	19.6	Rose red	102.0
Sulfadiazine	0.05	21.5	Rose red (orange tinge)	93.0
Sulfanilamide	0.05	15.0*	Orange red	133.3*
Sulfamerazine	0.025	20.0	Rose red	100.0
Sulfathiazole	0.025	18.9	Rose red (violet tinge)	105.9

*Approximate readings, as these solutions were too orange to match closely.

To 5 c.c. of the filtrate and to 5 c.c. quantities of appropriate standards are added 5 c.c. of the citric-p-toluenesulfonic acid or the citric-N/5 H_2SO_4 stabilizing solution* and 1 c.c. of 0.1 per cent sodium nitrite. After three minutes add 1 c.c. of the color reagent and again mix the contents of the tubes. A deep red color immediately develops and should be stable for at least an hour. The solution should not be turbid nor have a violet off-shade. The standard which most closely matches the unknown is set at 20 mm. in the colorimeter.

Five cubic centimeter amounts of the various standard solutions of the sulfonamides were subjected to analysis in this manner and then compared with each other in the colorimeter. The sulfamerazine preparation was set at 20 mm. The various colors and readings are given in Table I. It was found that the various red colors match each other reasonably well, except sulfanilamide which was too orange red. From the data in this table one can readily obtain the necessary correction factor as compared with sulfamerazine, for example; hence, only a single standard need be prepared, except in the case of sulfanilamide.

Sulfonamide Adsorption by the Protein-Tungstate Precipitates.—Sulfonamide adsorption in appreciable quantities during blood deproteinization is well recognized.[2, 3, 6] In the various procedures which utilize trichloroacetic acid or p-toluenesulfonic acid as blood protein precipitants, sulfonamide adsorption is minimized by increased acidity of the filtrates and blood dilution up to from 1 to 50.

In the following experiments various factors which could influence sulfonamide adsorption were studied. Five hundred cubic centimeters of blood containing 1 mg. of potassium oxalate per cubic centimeter were obtained from a donor. Five cubic centimeter portions were pipetted into flasks, and sufficient

*While the citric-p-toluenesulfonic acid solution was used in most of the experiments reported in this paper, many of the more vital experiments were checked with the citric-N/5 H_2SO_4 also. The values always checked closely, however. While the color obtained in the latter solutions was slightly more orange red, the actual amount of color was the same and the readings nearly identical. With sulfathiazole, however, clear solutions were obtained only with p-toluenesulfonic acid which dissolves the dye. Trichloroacetic acid can also be used but does not seem as satisfactory. If considerable quantities of tungstate ions are present in the filtrate, due to excess oxalate in the blood, even a large excess of citric acid does not protect against their interference in the reaction.

quantities of the standard sulfonamide solutions in N/11.4 H_2SO_4 were added in order to obtain a series of bloods ranging from 2.5 to 30 mg. sulfonamide per 100 c.c. of blood. Then sufficient N/11.4 H_2SO_4 was added to bring the amount of acid up to 40 c.c. in each case. After adding a drop of N/1 H_2SO_4, each flask was swirled gently for several minutes. Then 5 c.c. of 10 per cent sodium tungstate were added slowly with shaking. The flasks were shaken for several minutes. The contents were poured onto No. 1, 12.5 cm. Whatman filter papers.* The complete hemolysis, precipitations, and filtrations were carried out at approximately 18, 26, and 33° C., respectively. The filtrates were then analyzed for their sulfonamide content as previously outlined.

The results obtained from these experiments proved that sulfonamide adsorption is not only considerable but varies with the nature of the sulfonamide as well. However, the percentage recovery in the filtrates for any one sulfonamide is consistent and independent of its concentration in the blood. Hence, a factor can be used to obtain the correct value in each case. Thus, the percentage recovery at 26° C. for sulfanilamide is 88.3; sulfadiazine, 75.7; sodium sulfadiazine, 75.8; sulfamerazine, 75.8; sulfamethazine, 76.1; sulfapyridine, 74.4; sulfathiazole, 56.7; and sulfacetimide, 83.8. Similar values are obtained when 2, 3, and 4 c.c. of blood are deproteinized. The recovery values are slightly higher (from 2 to 6 per cent) when the bloods are deproteinized at approximately 33° C. and slightly lower (from 0.5 to 2 per cent) at 18° C. It is obvious that the degree of adsorption does not parallel sulfonamide solubility. Adsorption of conjugated or acetylated sulfonamides was not studied. Other experiments showed that dilution of blood up to from 1 to 40 does not result in complete recovery of the drug. This loss is not due to inhibitory factors in the tungstic acid filtrates nor do sulfonamide-free filtrates yield appreciable color blanks.

Carryer and Osterberg[7] found that 20 per cent of the sulfathiazole added to bile was adsorbed when filtrates were prepared with potassium hydroxide and zinc sulfate. Davis[8] has recently shown that the albumin of plasma appreciably binds the sulfonamides. In the present experiments it was also found that the protein content of blood may undergo considerable depletion, up to 35 per cent, before an appreciable decrease in sulfonamide adsorption is observed. In such cases, however, it is well to decrease the sodium tungstate by about 10 per cent to assure satisfactory filtrates. Also, 2 or 3 c.c. portions of sulfonamide-containing bloods were hemolyzed at different pH by adding an extra drop of sulfuric acid of varying strengths. The bloods were then deproteinized and analyzed in the usual manner. It was found, however, that but a slight decrease in sulfonamide adsorption (from 2 to 6 per cent) occurs as the pH of the filtrates drops from about 3.7 to 2.4.

*Several varieties of Whatman filter paper manufactured by W. & R. Balston, Ltd., England, including the acid treated No. 30 and 40 types and the semicrimped, qualitative filter paper made by Fisher Scientific Co., Pittsburgh, Pa., were found satisfactory. All yielded the same percentage sulfonamide recovery values. Likewise blood filtrates obtained by centrifugation also contained the same concentration of sulfonamides. However, the Reeve Angel's filter paper (H. Reeve Angel & Co., New York, N. Y.) was found quite unsatisfactory. Filtrates obtained with this paper, when subjected to analysis, always yielded turbid solutions and a violet off-shade.

TABLE II. RELATION OF COLOR INTENSITY TO AMOUNT OF SULFONAMIDE (CALIBRATION CURVES)

AMOUNT OF SULFONAMIDE (μG PER 2 C.C.)	READINGS ON THE KLETT-SUMMERSON PHOTOELECTRIC COLORIMETER FOR VARIOUS SULFONAMIDES WITH THE MODIFIED BRATTON-MARSHALL PROCEDURE					
	SULFANILAMIDE	SULFATHIAZOLE	SULFAPYRIDINE	SULFADIAZINE	SULFAMERAZINE	SULFAMETHAZINE
50.0	801	520	551	550	520	484
25.0	398	268	280	274	265	243
12.5	201	136	141	140	133	123
6.25	101	69	71	70	66	63
3.125	50	34	36	36	33	31
1.563	24.5	17	18	17	16	16

Photoelectric Adaptation of the Bratton-Marshall Method to Tungstic Acid Filtrates.—Two cubic centimeters of the filtrate, 2 c.c. portions of appropriate standard sulfonamide solutions, and 2 c.c. of distilled water are each diluted with 5 c.c. of the citric-p-toluenesulfonic acid or the citric-N/5 H_2SO_4 stabilizing solution (which has been diluted 1 to 1 with water). Add 0.5 c.c. of 0.1 per cent sodium nitrite. After three minutes add 0.5 c.c. of the sulfamate buffer solution. Mix, wait for three minutes, and add 0.5 c.c. of the color reagent. A deep red color develops immediately and remains clear and stable for at least an hour. Turbidity and a violet off-shade with consequent low readings indicate an excess of tungstic acid ions in the filtrate. The instrument is adjusted to zero reading with the water blank. The readings in the present experiments were made about three minutes after color development in a Klett-Summerson photoelectric colorimeter. It contained a green No. 54 filter which came with the instrument.

TABLE III. COMPARATIVE ANALYSIS OF SULFONAMIDE-CONTAINING BLOODS (MG. PER 100 C.C.)*

NUMBER	SULFONAMIDE	BRATTON-MARSHALL COLORIMETRIC PROCEDURE	ANALYSIS OF TUNGSTIC ACID BLOOD FILTRATES BY THE MODIFIED BRATTON-MARSHALL METHODS		HOSPITAL VALUES BY PHOTOELECTRIC BRATTON-MARSHALL METHOD
			COLORIMETRIC	PHOTOELECTRIC	
1	Sulfadiazine	28.5	28.9	29.6	28.0
2	Sulfadiazine	12.0	11.6	11.5	11.9
3	Sulfadiazine	9.0	8.9	8.7	8.7
4	Sulfadiazine	3.6		3.2	3.1
5	Sulfadiazine	1.1	1.3	0.8	1.0
6	Sulfathiazole	52.2	53.5	48.3	51.0
7	Sulfathiazole	24.0	24.1	24.6	21.0
8	Sulfathiazole	13.2	13.6	14.2	12.5
9	Sulfathiazole	6.0	6.5	6.5	
10	Sulfathiazole	3.2	2.8	2.9	3.2
11	Sulfapyridine	19.5	21.5	18.9	20.0
12	Sulfapyridine	9.6	10.3	9.3	10.0
13	Sulfapyridine	3.0	3.3	3.0	3.0
14	Sulfapyridine	1.7	1.4	1.9	1.5
15	Sulfamerazine	16.7	18.7	16.8	17.5
16	Sulfamerazine	10.5	10.6	10.1	10.7
17	Sulfamerazine	7.9	7.6	7.5	7.3
18	Sulfanilamide	19.0	18.8	19.0	19.4
19	Sulfanilamide	9.4	9.4	9.0	9.5
20	Sulfanilamide	3.2	3.3	3.1	3.5*

*Individual standards were used for each sulfonamide determination. The values obtained with the methods on tungstic acid filtrates were corrected for sulfonamide loss during deproteinization.

The blood values may also be obtained from a calibration curve obtained by analysis of a series of standard sulfonamide solutions as previously described. The data for a number of the more common sulfonamides, given in Table II, indicate that the relationship between the concentration of the various sulfonamides and the intensity of color is linear between 2 and 50 μg per 2 c.c. of solution. By proper correction for sulfonamide loss during deproteinization, values expressed as milligrams per 100 c.c. of blood may be obtained directly from the Klett-Summerson readings.

Comparative Analysis of a Series of Sulfonamide-Containing Bloods.—A number of bloods from patients undergoing sulfonamide therapy were obtained from the hospital laboratory. These bloods had been previously analyzed by the regular hospital laboratory staff, using the well-known Bratton-Marshall[1] photoelectric procedure. They were then analyzed by the new methods as adapted to tungstic acid filtrates and by the regular Bratton-Marshall[1] visual colorimetric procedure. The results are given in Table III and indicate that the values given by the various methods are in satisfactory agreement.

SUMMARY

The colorimetric methods of Bratton and Marshall[1] for blood sulfonamides have been adapted to tungstic acid (Folin-Wu) blood filtrates. Various factors which influence these methods have been studied. Comparative analyses of bloods from patients undergoing sulfonamide therapy were made by various procedures, and the values obtained were found to be in satisfactory agreement.

The author wishes to acknowledge the helpful cooperation of Dr. Marie Andersch and the various members of the University Hospital laboratory staff. The Winthrop Chemical Company, Inc., New York, N. Y., Schering Corporation, Bloomfield, N. J., Mead Johnson and Company, Evansville, Ind., and Sharp & Dohme, Inc., Glenolden, Pa., made generous gifts of sulfonamides for which the author is very grateful.

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A SIMPLY CONSTRUCTED METABOLISM CAGE FOR SMALL RATS

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IN CONNECTION with studies of the metabolism of some of the vitamins of the B group, it was necessary to collect urine quantitatively from a small rat, in such a way that there was no contamination of the urine by feces or food. It was also necessary to be able to measure the actual food consumption, either with ad lib. or with restricted feeding, and to feed small known amounts of food to the rat in such a way that all the food given could be consumed.

A Hopkins metabolism cage,* modified in such a way as to allow the rat to obtain small amounts of food quantitatively, was found to fulfill all these requirements.

The metabolism cage is illustrated in Figs. 1 and 2. As shown in Fig. 2, the cage proper, *A*, fitted with a feeding tube, *B*, and a water bottle, *C*, rests in a stemless filter funnel, *D*. A beaker, *E*, containing a small Erlenmeyer flask, *F*, fitted with a conical glass bulb, *G*, is placed so that the tip of the bulb is directly beneath the funnel.

The cage is made of wire or tin and may be of any appropriate size depending on the size animal to be used in it. The floor is made of 22 gauge galvanized wire mesh soldered on to the outside. The lid is made to fit over the outside, and a wire rest is attached for the water bottle, *C*, the glass tube from which is led through the lid and extends a few inches into the body of the cage. The hole cut out of the side of the cage for the feeding tube is 1½ inches in diameter, with the center approximately 1¼ inches from the floor. A copper wire about 10 inches long and bent so as to form a circular support for the feeding tube is soldered to the cage on both sides of this hole.

The feeding tubes, *B*, which are made from test tubes, are in two sizes: size A, from 0.94 to 0.95 inches in internal diameter, approximately 3 inches long; size B, from 1.08 to 1.12 inches in internal diameter, approximately 4.5 inches long.

The tubes are flanged as shown in Fig. 2 and are clamped in position by means of the wire support which can readily be bent into shape with the fingers. The tube must be held firmly.

The diameter of the opening of the funnel should be at least ⅝ inches to prevent blockage by solid material. The bulb, *G*, which is blown from soft glass tubing, is about 1½ to 2 inches in diameter and 2 inches high, with a stem from 1 to 1½ inches long fitting into indentations made in the neck of a 100 c.c. Erlenmeyer flask, *F*. The stem is ground into the indented neck of the

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*Ackroyd, H., and Hopkins, F. C.: Feeding Experiment With Deficiencies in the Amino-acid Supply. Arginine and Histidine as Possible Precursors of Purines, Biochem. J. 10: 551, 1916.

flask by means of emery powder and glycerin; in this way, the bulb is attached rigidly to the Erlenmeyer flask. The indentations in the neck allow the urine to run into the flask.

The distance between the funnel and the top of the bulb should be just sufficient to allow feces to fall away; if it is too great, liquid tends to splash off the bulb. The shape of the bulb is most important; it should be peaked, with

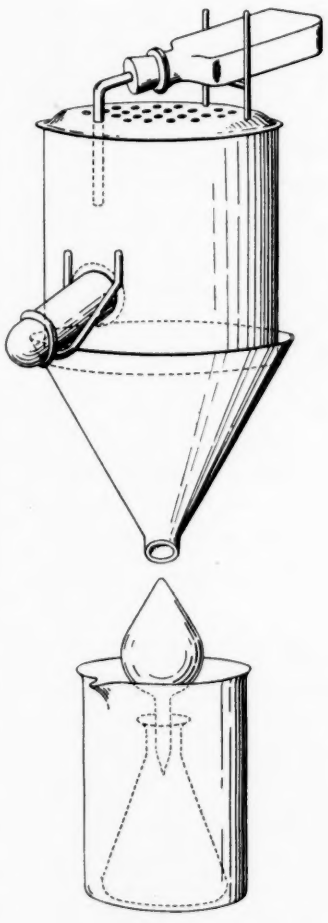

Fig. 1.

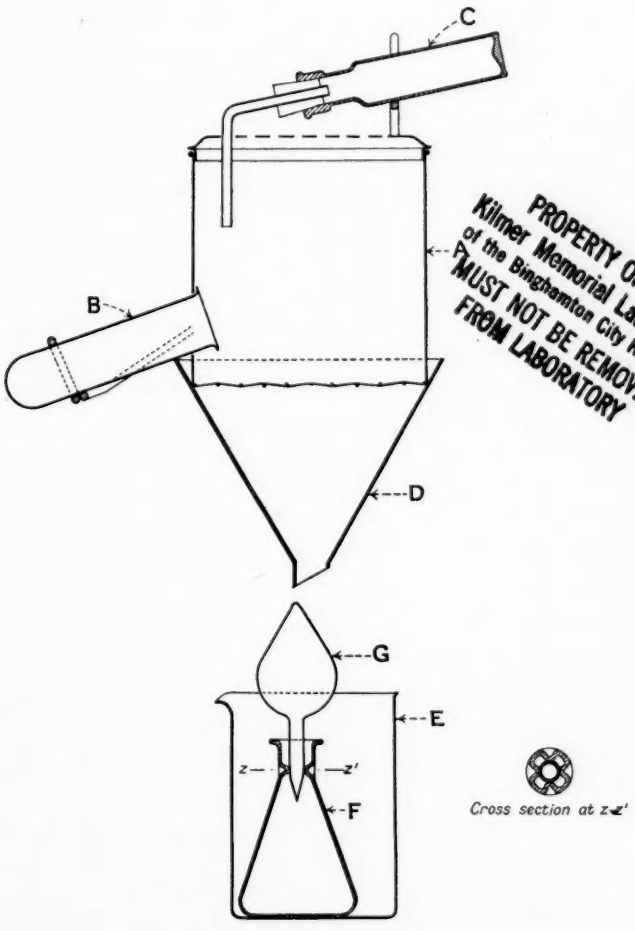

Fig. 2.

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FROM LABORATORY

Fig. 1.—Assembled cage.
Fig. 2.—Cross section of assembled cage through central axis.

steep but not vertical sides which should merge gradually into the stem so that liquid runs down the stem and does not drip from the bulb.

The dimensions of the feeding tubes should be such that the rat can reach the bottom of the tube, but only with difficulty. With such a tube, the rat is unable to remove food from the tube with its paws; if the tubes are made larger,

the rats invariably shovel the food into the cage with their front paws. It was found that size A was suitable for rats weighing from 20 to 40 grams, and size B was suitable for rats weighing from 40 to 80 grams. A larger feeding tube is necessary for rats weighing more than 80 grams.

In this type of cage, the nature of the food mixture given to the rats is important. If a dry powdery mixture is used, considerable scattering occurs, occasionally causing blockage of the funnel. To prevent this, such a powder should be mixed with water to form a thick mash. More suitable types of food mixtures are those prepared from sugar, oils, and casein. The large particle size of the sugar and the binding action of the oil both tend to prevent scattering. A mixture with a high fiber content is undesirable, because the bulk of the feces formed sometimes causes blockage at the mouth of the filter funnel.

We wish to express our appreciation to Mr. E. P. Bayliss, F.R.G.S., of the Survey Branch, Department of Interior, for drawing the diagrams for this paper.

AN IMPROVED METHOD FOR THE DETERMINATION OF PLASMA PROTHROMBIN

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INTRODUCTION

MANY methods have been employed for the clinical determination of the prothrombin concentration of plasma. All of these methods are based upon the principle that in the presence of an optimum calcium ion concentration and an excess of thromboplastin the coagulation time of the plasma is related to the prothrombin concentration.

This relationship, however, has been found to be hyperbolic rather than linear, so that the coagulation time is an insensitive measure of prothrombin concentration when the latter is high but a sensitive measure when it is low. For this reason the original Quick method, which employed undiluted plasma, can be considerably improved in accuracy by dilution of the test plasma with prothrombin-free plasma.

It was the purpose of the present investigation to develop a satisfactory diluent for plasma, a stable thromboplastin preparation, and a procedure for the determination of prothrombin to be used for clinical and experimental purposes. Although this investigation was carried out using dog's plasma, the same procedures can be applied to human plasma.

METHODS

Preparation of Stable Thromboplastin.—In all the methods used to determine prothrombin concentration of blood, the coagulation time is notably influenced by the activity of the thromboplastin suspension. A stable and uniform preparation of this reagent is therefore necessary to render a technique or method reliable.

The concentration of the thromboplastic substance in the brains of different animals of the same species is variable; for example, when three different powdered brains of the same animal species were tested against the same dilution of dog's plasma, the resulting coagulation times were 40, 50, and 32 seconds, respectively.

Quick prepares his thromboplastin by shaking brain powder in normal saline solution and then centrifuging it or allowing it to settle. The supernatant, which is the reagent to be used, has a variable activity because of the influence of temperature, intensity of shaking, and completeness of settling or centrifuging.

It was found that these difficulties could be avoided by employing a suspension of the total brain powder made without centrifuging.

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Preparation of Rabbit Brain Powder: The rabbits are killed by a 20 c.c. intravenous injection of air. Then the meninges are carefully removed and the brains repeatedly triturated with anhydrous acetone in order to rapidly remove all traces of water. The acetone is evaporated at room temperature, and the drying is finally completed in vacuo over sulfuric acid. The dehydrated brain tissue (from 50 to 100 Gm.) is finely pulverized in a mortar so as to obtain a uniform powder. This powder is placed in glass ampules in 0.15 Gm. fractions; before sealing, the ampules are placed in the desiccator in order to remove humidity that may have been absorbed during the weighing process. They are then closed in an atmosphere of nitrogen. Brain powder prepared in this way retains its activity for as long as five months.

Preparation of the Powdered Brain Suspension (Thromboplastin): The contents of one ampule (0.15 Gm. of brain powder) are again finely pulverized in a mortar. By adding 0.3 c.c. of a 0.85 per cent sodium chloride solution, a homogenous paste is obtained and is diluted by four successive additions of 2 c.c. of sodium chloride solution. The dilution is brought up to 50 c.c. by normal saline solution, and the suspension is then shaken and heated for ten minutes at 37° C. to obtain a uniform temperature throughout. The suspension must be agitated by shaking or by blowing down the pipette before taking a sample.

TABLE I. INFLUENCE OF CONCENTRATION OF BRAIN POWDER ON COAGULATION TIME

BRAIN POWDER (GM./100 C.C.)	COAGULATION TIME OF DOG'S PLASMA (SEC.) DILUTION			
	1/0	1/10	1/20	1/50
0.6	10.5	23.5	34.5	78.5
0.3	*10.5*	*18*	*28.5*	*40*
0.15	12	19	27.5	47
0.075	16	23.5	32	57.5

TABLE II. INFLUENCE OF TEMPERATURE ON ACTIVITY OF 0.3 PER CENT BRAIN POWDER SUSPENSION

TEMPERATURE (° C.)	COAGULATION TIME OF DOG'S PLASMA (SEC.) DILUTION			
	1/0	1/10	1/20	1/50
37	*11.5*	*16*	*26.5*	*36*
45	12	18.5	26.5	40.5
60	22	36.5	51	83
100	23.5	38	55.5	91.5

The suspension prepared in this way has 0.3 Gm. of powdered rabbit brain in 100 c.c. of the suspension in contrast to Quick's preparation which contains the supernatant from 3 Gm. of brain powder in 100 c.c. of solution.

We have found that a higher concentration of the brain powder considerably prolongs the coagulation time (Table I). This was first shown by Aggeler and Lucia. The mechanism of the anticoagulant effect of an excess of thromboplastin is not understood. We also have found that a temperature of 37° C. is the most convenient for obtaining an active, stable, and uniform suspension. A higher temperature prolongs the coagulation time as can be seen in Table II.

This confirms the results obtained by Quick relative to the influence of the temperature on the activity of the thromboplastin suspensions.

Stability of the Thromboplastin: Stock rabbit brain powder prepared by the previously described method has been found to retain its full activity for periods as long as five months. Table III shows the results of periodic assays of such a preparation. The assay consisted of using the powder to determine the plasma prothrombin level of normal dogs maintained under uniform conditions and used for no other purpose. The results reveal random fluctuations in prothrombin level but no trend in a downward direction indicative of progressive loss of activity of the thromboplastin preparation. The random fluctuation is due, in large part, to the use of normal dog plasma as a source of prothrombin; however, had a uniform preparation of pure prothrombin been available, the random fluctuation could have been minimized.

TABLE III. STABILITY OF THROMBOPLASTIN SUSPENSION TESTED ON PLASMA OF CONTROL DOGS

DOG 133		DOG 134	
DAYS	PROTHROMBIN (%)	DAYS	PROTHROMBIN (%)
1	83	1	108
2	87	2	98
3	81	3	107
4	88	4	94
5	97	5	95
7	100	7	105
10	86	12	114
15	95	17	105
20	90	21	101
25	90		
26	104		
29	92		
36	95		
45	90		
Average	88.4	Average	103
Range	81 to 104	Range	94 to 114

Development and Preparation of a Satisfactory Diluent.—A satisfactory diluent for plasma in the determination of prothrombin must not interfere in any way with the coagulation of the plasma which it dilutes. This means that the diluent must contain no prothrombin activity itself, and it must not influence the prothrombin level, fibrinogen level, or pH of the diluted plasma.

The influence on coagulation time and clot formation of alteration in fibrinogen level and pH has been demonstrated experimentally. In Table IV it

TABLE IV. EFFECT OF FIBRINOGEN CONCENTRATION ON COAGULATION TIME

OXALATED PLASMA (C.C.)	OXALATED SERUM (C.C.)	DILUTION	THROMBIN* 1/10 (C.C.)	COAGULATION TIME (SEC.)
0.1	-	1/0	0.1	17.1
0.05	0.05	1/2	0.1	21.4
0.02	0.08	1/5	0.1	26.3
0.01	0.09	1/10	0.1	95

Dilutions of 1/5 and 1/10 yield friable clots.
*Thrombin prepared according to Eagle technique.

TABLE V. INFLUENCE OF pH ON COAGULATION TIME

TUBE	pH	COAGULATION TIME (SEC.) DILUTION 1/20
1	5.40	33.5
2	6.55	21
3	7.15	20
4	7.85	21.5
5	8.2	22
6	9.4	23
7	10.1	No coagulation at 2 min.
8	10.7	No coagulation at 2 min.

TABLE VI. COMPARISON OF PROTHROMBIN ADSORBENTS

SUBSTANCES	pH OF SUSPENSION	pH OF PLASMA	COAGULATION TIME
SO_4Ba	7.2	7.8	No coagulation
CO_3Ba	8.2	8.3	No coagulation
CO_3Zn	7.3	8.3	No coagulation
$(OH)_2Zn$	8.5	8.7	4 min.
$(OH)_3Al$	8.7	8.6	No coagulation
$(OH)_2Mg$	10.1	9.1	19 min.
$(CO{\cdot}O)_2Ca$	7.2	8.2	No coagulation
$(OH)_3Fe$	-	-	No coagulation
SO_4Pb	2.73	7.6	4 min.
Talcum	-	-	20 sec.
Kaolin	-	-	45 sec.

is shown that dilution of plasma with normal serum, which reduces the fibrinogen level, markedly delays the coagulation time of plasma. In Table V it also is shown that beyond the pH limits of from 6.0 to 8.0 coagulation is markedly interfered with. This confirms the previous work of Mertz and Owen.

Normal plasma from which prothrombin has been completely removed would appear to offer the best source of a satisfactory diluent. A number of investigators have used various procedures for removing prothrombin from solution, including adsorption on magnesium hydroxide (Fuchs), aluminum hydroxide (Quick), tricalcium phosphate (Bordet and Delange), and barium sulfate (Dale and Walpole). We made a comparative study of these and other agents with respect to their effectiveness in removing prothrombin from normal plasma and their pH. The substances studied are shown in Table VI. All of them were prepared by precipitation, using a large volume of water, and then they were washed thoroughly. The precipitate obtained was suspended in a small volume of water and tested as an adsorbent of prothrombin. In every instance the procedure was as follows: 0.2 volume of the suspension was added to 1 volume of plasma; the mixture stood for ten minutes at 37° C., after which it was centrifuged for ten minutes at 3,000 r.p.m. The coagulation time of the supernatant was determined by adding 0.2 c.c. of the thromboplastin-calcium mixture to 0.1 c.c. of the supernatant plasma.

Among the substances tested by us, barium sulfate was the most satisfactory because of its neutrality, its almost complete insolubility, and the facility with which it can be removed by centrifuging. Furthermore, barium sulfate is much more active than the other substances tested regarding its ability to prevent coagulation. In fact, a sample of plasma centrifuged after being in contact

for ten minutes, at room temperature, with 5 per cent barium sulfate suspension, does not clot when thromboplastin and calcium are added. The plasma so obtained is completely clear and has the same pH as normal plasma.

The barium sulfate in the dried form also adsorbs prothrombin but yields plasma with some turbidity which is not cleared by repeated centrifuging.

Preparation of a 30 Per Cent Barium Sulfate Suspension: This suspension is obtained from barium chloride and sulfuric acid. Thirty-five grams of crystallized barium chloride are weighed and dissolved in 500 c.c. of water; while shaking, a solution of 11 c.c. of sulfuric acid (specific gravity, 1.84) in 500 c.c. of water is added. This is filtered or centrifuged and then washed with distilled water until there is no further acid reaction to methyl red. The precipitate is suspended in 100 c.c. of distilled water. The adsorbing property of this suspension diminishes if it becomes acid. It may be renewed by washing with distilled water until a neutral reaction is obtained. The original volume is then restored by the addition of distilled water.

Preparation of Diluent Plasma: To 1 volume of oxalated plasma 0.2 of a volume of barium sulfate suspension is added, shaken, and warmed for ten minutes at 37° C. and then centrifuged at high speed (3,000 r.p.m.) and decanted. We call this plasma barium plasma to differentiate it from plasma treated by other adsorbents. It should not coagulate on adding thromboplastin and calcium, for it has no prothrombin. The barium plasma is more efficacious as a diluent plasma than the alumina plasma of Quick. In Table VII is presented the results of an experiment which demonstrates that the coagulation times are shorter by using barium plasma as a diluent as compared with the alumina plasma. This is due to the fact that some aluminum hydroxide remains suspended and adsorbs prothrombin from the normal plasma.

TABLE VII. COMPARATIVE STUDY OF BARIUM PLASMA AND ALUMINA PLASMA

HUMAN PLASMA (C.C.)	HUMAN (DILUTED) PLASMA (C.C.)	COAGULATION TIME USING BARIUM PLASMA AS A DILUENT (SEC.)	COAGULATION TIME USING ALUMINA PLASMA AS A DILUENT (SEC.)
0.02	0.08	25	32
0.01	0.09	35	92
0.005	0.095	57	No coagulation

Apparatus, Reagents, and Technique of Determination.—The apparatus required consists of a water bath, 0.2 c.c. graduated pipettes divided to 0.001, Kahn tubes, test tubes, and a stop watch.

Fig. 1 shows the water bath used by us. This is composed of a Pyrex beaker of 1 L. capacity (1) and a vessel of bronze (2), 6 cm. in height and 5 cm. in diameter with holes at its base to permit the free circulation of water. The tubes are placed in the bronze vessel and kept at a constant temperature. A second perforated bronze vessel (3), 6 cm. long and 1.5 cm. in diameter, is attached to a flexible bronze wire of 3 mm. in diameter; this wire permits the vessel to be placed in any position desired by the operator. The temperature of the bath may be regulated by a flame, the siphon (4), and the thermometer (5).

The test tube (6) which contains the thromboplastin-calcium-mixture and the pipette (7) are kept in the bath at 37° C. in order to avoid any change in temperature of the plasma.

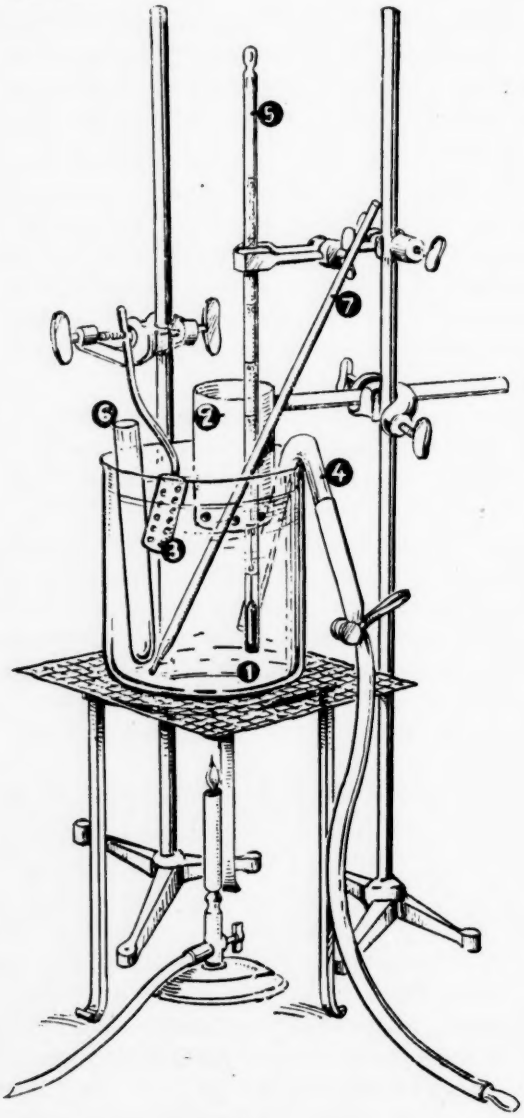

Fig. 1.

The reagents required consist of 0.1 M. sodium oxalate (1.34 Gm. per 100 c.c.), 0.025 M. calcium chloride (0.28 Gm. per 100 c.c.), and the 0.3 per cent thromboplastin and 30 per cent barium sulfate suspensions previously described.

Technique of Determination:

1. Blood extraction. One volume of sodium oxalate is added to 9 volumes of blood. The mixture is centrifuged, and the plasma thus obtained is divided

into two portions. One portion is used in the preparation of barium plasma and the other kept as plasma to be tested.

2. Preparation of the barium plasma (see foregoing).

3. Preparation of thromboplastin-calcium mixture. One volume of thromboplastin suspension is added to 1 volume of 0.025 M. $CaCl_2$ solution.

4. Different amounts of barium plasma and plasma to be tested are allotted to different test tubes according to the schedule shown in Table VIII.

TABLE VIII

TUBE	P.Ba.* (C.C.)	P.E.† (C.C.)	P.E. 1/10 (C.C.)‡	DILUTION	P.E. % PROTHROMBIN	THROMBO-Ca (C.C.)	COAGULATION TIME (SEC.)
1 + 2	-	0.1	-	1/0	100	0.2	
3 + 4	-	-	0.1	1/10	10	0.2	
5 + 6	0.05	-	0.05	1/20	5	0.2	
7 + 8§	0.08	-	0.02	1/50	2	0.2	

*P.Ba., Barium plasma.

†P.E., Plasma to be tested.

‡The 1/10 P.E. is prepared by adding 0.05 c.c. of plasma to be tested to 0.45 c.c. of barium plasma.

§When testing human blood 0.07 c.c. of P.Ba. and 0.03 c.c. of P.E., 1/10 are used, thus giving a dilution of 1/33.3.

TABLE IX. COAGULATION TIME OF DIFFERENT DILUTIONS OF PLASMA OF NORMAL DOGS

DOG	COAGULATION TIME (SEC.) DILUTION 1/0	1/10	1/20	1/50
507	10.5	15.5	26.5	38
509	11.5	16.5	24.5	39
516	11	14.5	25	37
519	10.5	15	23	36.5
520	10	14.5	23.5	36
506	11.5	15	24.5	38.5
508	11	15.5	23.5	37
510	11	14.5	24	38
511	10.5	14	22.5	36.5
512	11.5	15.5	23.5	38
521	11	16	24	36.5
Average	10.9	15.1	24	37.4

The determinations at each dilution are made in duplicate. The barium plasma is always measured out first into each tube to be used so as to avoid possible barium plasma contamination with prothrombin plasma. Then, undiluted plasma to be tested is added and finally diluted plasma to be tested (1/10). Special attention must be paid to keeping the outside of the pipettes dry and to pipetting aliquots into the bottom of the tubes, since small amounts of plasma could remain on the sides of the test tubes. It is also advisable to use a different pipette for each plasma.

The tubes with the measured plasma, the mixture of thromboplastin and calcium, and the 0.1 c.c. pipettes are placed in the 37° C. water bath. Each tube (separately) will then receive 0.2 c.c. of the thromboplastin-calcium mixture, and the coagulation time is determined by means of the chronometer from

the moment the reagent has been added.* The thromboplastin-calcium preparation should be mixed each time by blowing down the pipette before starting the measurements.

The Relationship Between Coagulation Time (Prothrombin Time) and Prothrombin Concentration.—In 1937 Quick and Leu diluted normal human and rabbit plasma with prothrombin-free plasma, prepared by the $Al(OH)_3$ method, in order to obtain various relative concentrations of prothrombin. They were able to show that in the presence of optimum calcium and excess thomboplastin the coagulation time and relative prothrombin concentration bear an inverse hyperbolic relationship to one another.

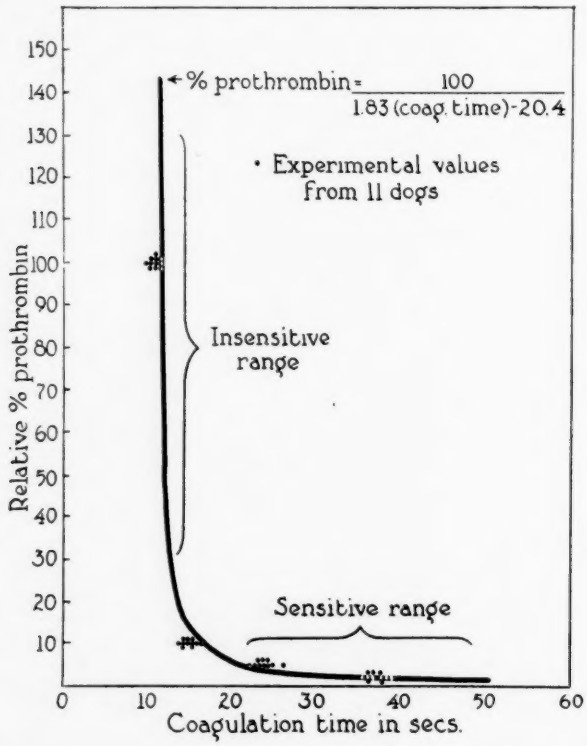

Fig. 2.

These experiments have been repeated using the plasma of eleven normal dogs diluted with prothrombin-free plasma prepared according to the procedure described herein. The percentages of prothrombin and the observed coagulation times are presented in Table IX, and plotted in Fig. 2.

On examining the graph of Fig. 2 it is evident that:

1. A change in prothrombin concentration from 100 per cent to 50 per cent corresponds to a nearly imperceptible change in coagulation time.

*Formation of foam must be avoided in order not to miss the formation of the clot. This is accomplished by adding the thromboplastin-calcium mixture in the following manner: the pipette is placed at the bottom of the tube, and then its contents are blown out; at this moment the stop watch is started and the pipette is removed while continuing the blowing so that the stream of air can break any foam that may have been formed.

2. On the other hand a change in prothrombin from 50 per cent to 2 per cent corresponds to an easily measurable change in the coagulation time.

3. The error due to the measurement of coagulation time is overwhelming if the coagulation time is less than that corresponding to 100 per cent of prothrombin concentration.

Let us consider an example:

1. A tube with 100 per cent of prothrombin coagulates in 11.7 sec.
2. A tube with 50 per cent of prothrombin coagulates in 12.2 sec. } Diff. = 0.5 sec.
3. A tube with 2 per cent of prothrombin coagulates in 38.5 sec. } Diff. = 26.3 sec.

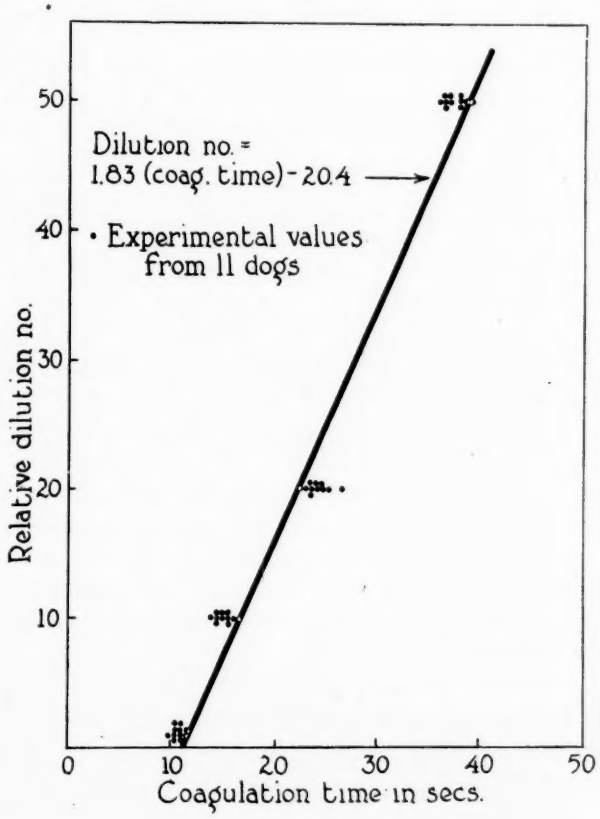

Fig. 3.

The results clearly show that the estimation of concentration using undiluted plasma is inaccurate and that the plasma must, therefore, be diluted in order to reach the sensitive portion of the curve in which significant variation in the coagulation time occurs with small variations in the concentration of prothrombin. For this reason dilutions of 1/10, 1/20, and 1/50 are recommended.

If the relationship between per cent prothrombin and coagulation time, as shown in Fig. 2, is truly a hyperbola, the relationship between the reciprocal of the per cent prothrombin and the coagulation time must be a straight line.

the moment the reagent has been added.* The thromboplastin-calcium preparation should be mixed each time by blowing down the pipette before starting the measurements.

The Relationship Between Coagulation Time (Prothrombin Time) and Prothrombin Concentration.—In 1937 Quick and Leu diluted normal human and rabbit plasma with prothrombin-free plasma, prepared by the $Al(OH)_3$ method, in order to obtain various relative concentrations of prothrombin. They were able to show that in the presence of optimum calcium and excess thomboplastin the coagulation time and relative prothrombin concentration bear an inverse hyperbolic relationship to one another.

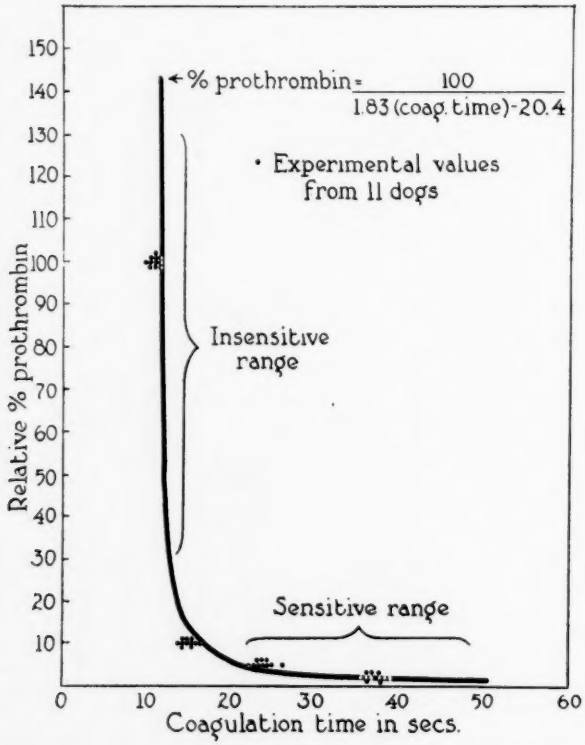

Fig. 2.

These experiments have been repeated using the plasma of eleven normal dogs diluted with prothrombin-free plasma prepared according to the procedure described herein. The percentages of prothrombin and the observed coagulation times are presented in Table IX, and plotted in Fig. 2.

On examining the graph of Fig. 2 it is evident that:

1. A change in prothrombin concentration from 100 per cent to 50 per cent corresponds to a nearly imperceptible change in coagulation time.

*Formation of foam must be avoided in order not to miss the formation of the clot. This is accomplished by adding the thromboplastin-calcium mixture in the following manner: the pipette is placed at the bottom of the tube, and then its contents are blown out; at this moment the stop watch is started and the pipette is removed while continuing the blowing so that the stream of air can break any foam that may have been formed.

2. On the other hand a change in prothrombin from 50 per cent to 2 per cent corresponds to an easily measurable change in the coagulation time.

3. The error due to the measurement of coagulation time is overwhelming if the coagulation time is less than that corresponding to 100 per cent of prothrombin concentration.

Let us consider an example:

1. A tube with 100 per cent of prothrombin coagulates in 11.7 sec.
2. A tube with 50 per cent of prothrombin coagulates in 12.2 sec. } Diff. = 0.5 sec.
3. A tube with 2 per cent of prothrombin coagulates in 38.5 sec. } Diff. = 26.3 sec.

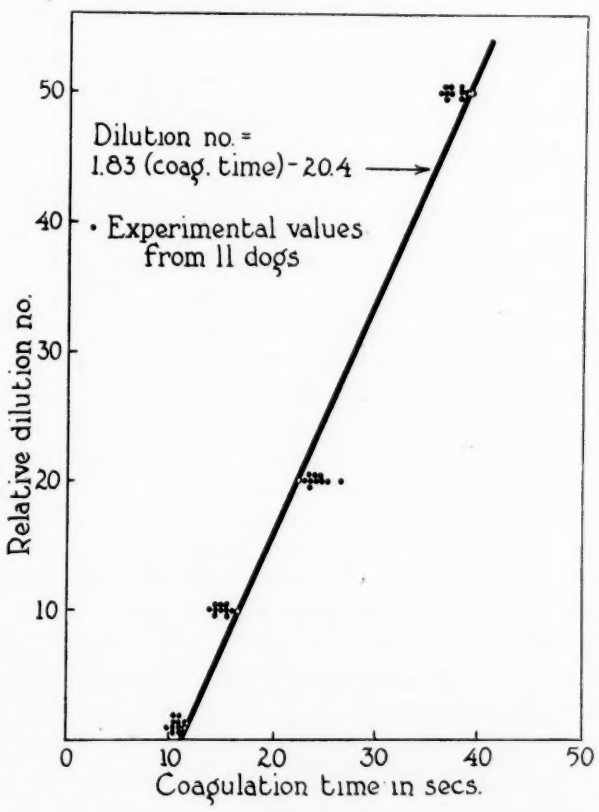

Fig. 3.

The results clearly show that the estimation of concentration using undiluted plasma is inaccurate and that the plasma must, therefore, be diluted in order to reach the sensitive portion of the curve in which significant variation in the coagulation time occurs with small variations in the concentration of prothrombin. For this reason dilutions of 1/10, 1/20, and 1/50 are recommended.

If the relationship between per cent prothrombin and coagulation time, as shown in Fig. 2, is truly a hyperbola, the relationship between the reciprocal of the per cent prothrombin and the coagulation time must be a straight line.

In Fig. 3 $\frac{100}{\%\text{ prothrombin}}$ is plotted against the coagulation time. It can be seen that the relationship is essentially linear.

The equation which best fits the data of Fig. 3 was found to be:

$$\frac{100}{\%\text{ prothrombin}} = 1.83\text{ (coagulation time)} - 20.4 \qquad (1)$$

This equation is plotted in Fig. 3 as the straight line. The corresponding equation for the hyperbola plotted in Fig. 2 is obtained by rearrangement of the preceding equation as follows:

$$\%\text{ prothrombin} = \frac{100}{1.83\text{ (coagulation time} - 20.4} \qquad (2)$$

The values of the constants in this equation vary slightly with different stock preparations of thromboplastin and vary considerably for normal plasmas from different animal species. For human plasma the following values have been obtained:

$$\%\text{ prothrombin} = \frac{100}{0.555\text{ (coagulation time)} - 8.24} \qquad (3)$$

Calculation of Results: The procedure for the determination of the plasma prothrombin level described herein yields a series of coagulation times for the several dilutions of plasma employed. It is desirable to convert these three figures into a single value representing prothrombin concentration rather than coagulation time. Since the absolute prothrombin concentration is unknown, it must be expressed in terms of percentage of normal. This requires the establishment of normal standards. We have used the following procedure for this purpose. Using a stock preparation of thromboplastin, determination of the coagulation time of many samples of normal plasma at various dilutions are made by the method described. This yields data of the kind presented in Table IX. These data are then plotted as in Fig. 3, for the purpose of obtaining the best fitting linear equation. This equation, when converted to the hyperbolic form, is the standard equation.

When determinations are made on unknown plasmas, the observed coagulation times at the various dilutions are used to calculate the percentage prothrombin from the standard equation. These values are then expressed as a percentage of the standard values at these same dilutions. Expressed in a formula, this becomes:

$$\%\text{ normal prothrombin} = \frac{\text{Observed \% prothrombin at a given dilution}}{\text{Standard \% prothrombin at the same dilution}} \times 100$$

TABLE X

TUBE	DILUTION	OBSERVED COAGULATION TIME	% OBSERVED PROTHROMBIN	% STANDARD PROTHROMBIN	% NORMAL PROTHROMBIN
1	1/10	18	7.97	10	79.7
2	1/20	24	4.25	5	85.0
3	1/50	46.5	1.54	2	77.0
Average % normal prothrombin					80

If three dilutions are employed, the resulting three values are averaged. An example of such a calculation is shown in Table X, based upon Equation 2 as the standard equation.

SUMMARY

1. A method is described for obtaining a preparation of thromboplastin from rabbit brains which retains its activity for at least five months.

2. A method is described for preparing prothrombin-free plasma by adsorption on barium sulfate.

3. Because of the inverse hyperbolic relationship between prothrombin concentration and coagulation time, the accurate determination of prothrombin in plasma requires dilution of the latter with prothrombin-free plasma.

4. An accurate method for determining plasma prothrombin based upon the foregoing findings is described.

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ANTISHEEP AMBOCEPTOR PRODUCTION WITH ELIMINATION OF RABBIT SHOCK

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Anne R. Bourke, B.S.,† Washington, D. C.

THE investigation herein described was begun in 1940 at the Division of Serology, Army Medical School, Washington, D. C., and completed in 1941. At that time this Division was making all the serologic reagents for the Regular Army. Therefore, anything hindering quantity production was a serious waster of time.

Chief among the hindrances were the heavy losses of rabbits by shock and, too often, the presence of agglutinogens or precipitins in the finished amboceptor. Everyone who has had to make amboceptor in any large amount knows the considerable losses of rabbits after the second dose of cells. If this dose is made large in the effort to produce amboceptor of a high titer, losses are inevitable. If the second dose is lowered, to escape fatal shock, the amboceptor produced is usually of too low a titer to be serviceable. These faults were inherent in the injection of the hemoglobin fraction of the sheep cells. Since Vedder[1] and Hadjopoulos[2] had proved that injection of cell stroma after laking would produce amboceptor, we decided to try some modification of their methods which would give a greater yield of cell stroma. The procedure we adopted has been used with perfect success in the Division of Serology for the past five years without the loss of a single rabbit. Since this method has been successfully tested by time, both in our hands and by others, we have felt it wise to offer it for wider use.

Two methods were tried, one by laking the cells with 0.4 per cent salt solution and the other by using amboceptor and complement to attain that end. Both were successful in that they eliminated all signs of shock in the rabbits and produced amboceptor of a high titer in ten days. The amboceptor-complement method produced nearly twice the amount of cell stroma as did the 0.4 per cent saline. Therefore, we adopted the former.

In following the procedure given in the next section, it is stressed that the sheep cells must be processed the same day on which the rabbits are to be injected. The cell stroma does not keep well in the refrigerator. We timed our cell preparation so that we could inject our rabbits immediately, as a rule, but it seemed just as effective to prepare them in the morning and inject them early the same afternoon.

PROCEDURE

Materials.—

1. Fresh washed, packed sheep cells.
2. Complement (pooled serum from twenty guinea pigs).

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3. Amboceptor (titer of 1:2000 or higher).

4. Salt solution, 0.85 per cent.

5. Graduated centrifuge tubes, 15 c.c. capacity.

Method.—

1. To 1.0 c.c. of washed and packed sheep cells add 1.0 c.c. of complement and 0.3 c.c. of undiluted amboceptor.

2. Incubate at 37° C. for fifteen minutes.

3. Dilute to 15 c.c. with 0.8 per cent salt solution, inverting gently once or twice to mix.

4. Centrifuge for five minutes.

5. Draw off supernatant fluid and set it aside.

6. Add 15 c.c. salt solution, mix gently, and combine with the supernatant set aside in step 5. This completes the hitherto incomplete hemolysis.

7. Centrifuge at high speed for thirty minutes.

8. Draw off and discard the supernatant fluid.

9. Add 15 c.c. of 0.85 per cent saline, mix gently, and centrifuge at high speed for thirty minutes. This removes all remaining traces of complement and amboceptor.

10. Draw off and discard the supernatant fluid. The residue of cell stroma is a translucent, pinkish- or light orange-colored mass, only barely distinguishable from the fluid. Care must be taken in all pipetting so that as little cell stroma as possible is wasted.

11. Make up the packed cell stroma to 1.0 c.c. with 0.85 per cent saline and mix gently.

This forms the initial dose for one rabbit. The second dose, given on the fifth day, is 2.0 c.c. The third dose, given on the tenth day, is 3.0 c.c.

It is not advisable to try to handle the sheep cells in amounts larger than 1.0 c.c. at a time. It is found that using larger amounts invariably produces relatively less stroma. As our doses are governed by the amount of original sheep cells, rather than by the final amount of stroma recovered, it is most desirable to recover the maximum yield possible.

The amount of washed and packed sheep cells to be obtained at the start depends, of course, on the number of rabbits to be injected. Thus, if five rabbits are to be treated, 5 c.c. of sheep cells are to be obtained for the first dose, 10 c.c. for the second, and 15 c.c. for the third. Processing fifteen separate centrifuge tubes at a time takes a little longer than processing one alone.

The injection is always made into the marginal ear vein.

Titers of from 1:4,000 to 1:10,000 are obtained after the second dose, that is, on the tenth day. These increase only slightly after the third dose, that is, on the fifteenth day, to from 1:5,000 to 1:12,000. The third dose is rarely needed.

Sex, breed, and age of rabbits apparently play no part in the titer of the amboceptor produced.

Emphasis is laid on the following facts in relation to this method:

1. During five years of use with over 500 rabbits, not a single rabbit has died from shock.

2. Satisfactory amboceptor is produced in ten days, with only a slight rise by the fifteenth day; in either case this is a great saving of time.

3. Titers produced are high compared to those produced by the packed, washed cell method. This is a far from negligible factor, as dilution to an acceptable 1:4,000 strength gives a much greater final yield.

4. It may be thought that the necessary preparation of the sheep cells is an unduly cumbersome and slow process. Actually it takes about ninety minutes, no matter how many tubes are used. This is a negligible factor in consideration of the increased yield and high titer of the product and the saving in rabbits and in time devoted to injection.

5. Finally, amboceptor thus produced gave identical results with standard commercial amboceptors made in the usual way when parallel tests were run.

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A RAPID METHOD FOR DRYING OXALATE SOLUTIONS

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THE preparation of oxalate tubes for collecting and preventing the clotting of blood is in many laboratories and offices a problem of great concern. Several books on clinical procedures recommend that tubes containing the oxalate solution be laid flat on a hot plate, rotated, and the contents allowed to splatter. Such treatment may cause decomposition of the oxalate preparations. This is especially true of oxalate anticoagulants made with ammonium and potassium oxalate. Directions usually given with such a mixture specify that the solution should be spread in a film over the lower walls and dried in an oven at not over 50° C. In addition to being a long, tedious process, such drying usually results in the formation of large crystals that of course go into solution slowly.

We have for some time been using a technique that we feel is most rapid and yet gives oxalate tubes with a thin film over the entire inner surface. In addition, there is no danger of oxalate breakdown by excessive heat.

The technique reported is extremely simple and requires only equipment available in any laboratory. The method is essentially that of lyophilization. The tube to be dried is stoppered with a one-hole soft rubber stopper which contains a short glass tube of a maximum size compatible with the size of tube used. The glass tube is connected to a good grade water pump by flexible rubber tubing heavy enough to withstand the negative pressure of the pump. Adjacent to such a setup should be a burner or hot plate to maintain a beaker of water at from 80 to 100° C.

DRYING OXALATE SOLUTIONS

Drying of the oxalate is accomplished by connecting the tube to be dried to the vacuum system and putting a maximum negative pressure on the system. The tube may now be held in a horizontal position by the left hand. Repeated and rapid snapping blows to the lower part of the tube with the right hand will distribute the oxalate over the entire inner surface of the tube. The tube should occasionally be dipped in the container of hot water to facilitate the evaporation of the water in the tube.

Test tubes or small flasks or bottles containing as much as from 1 to 1½ c.c. of oxalate solution may be drier in not over 2 or 3 minutes by this technique.

If a mechanical vacuum pump is available and provision is made for trapping the water that is distilled, even more rapid drying may be accomplished.

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BOOK REVIEWS

A. A. A. S. Research Conference on Cancer. Edited by *Forest Ray Moulton.* Publication of the American Association for the Advancement of Science, Smithsonian Institution Building, Washington, D. C. Science Press Printing Company, Lancaster, Pa., 1945. Price $4.50. Cloth with 333 pages.

The first A. A. A. S. monograph on cancer research was published in 1936 and was entitled *Some Fundamental Aspects of the Cancer Problem.* The second volume, *A. A. A. S. Research Conference on Cancer,* like the first volume, records in a measure the status of cancer research in 1944. The conference papers are divided into five groups as follows: on the virus approach, on carcinogenesis, on enzymes, on diets, and on chemotherapy.

The first article is on Virus Infection as an Etiologic Agent of Cancer by F. Duran-Reynals and E. W. Shrigley. This is an interesting paper on the possible role of viruses as causative agents of cancer; the authors compare and discuss the viruses that cause cell destruction or cell proliferation. The similarity of both necrotizing and neoplastic infections induced by viruses are compared with respect to the influence of genes, hormones, and physical and chemical agents on their activity as well as factors associated with age, as in cancer. A most interesting phase of this paper is the section on the viruses in relation to the effects they produce with respect to the age and resistance of the host. The section on the fundamental properties of viruses in relation to cancer in which the latency, variation, and masking of viruses are discussed is of particular interest to those in the cancer field.

The second paper is on the Virus Production of Mammalian Tumors by A. Taylor and A. Kynette. In this paper, the authors describe completely their work on the transmission of mammary tumors in mice from cell-free filtrates attained by use of the yolk sac technique for growing tumor tissue. Both Berkefeld filtrates and dried material from tumor-bearing eggs were capable of producing malignant tumors when injected into mice. However, reproducible procedures had not yet been developed, and the number of results obtained with Berkefeld filtrates was only positive in three experiments out of sixty, and the tumors produced as being of viral origin are open to question according to the following papers: Attempts to Demonstrate a Virus-like Principle in Mammalian Tumors by the Yolk Injection Technique by W. R. Bryan, H. Kahler, and V. T. Riley; Yolk Sac Cultivation and Virus Induction of Malignant Tumors by F. R. Heilman; and Virus Induction of Mammalian Tumors by G. H. Twombly.

H. Kahler and W. R. Bryan in the next paper, Characterization and Separation of Some Macromolecular Components in Tumors and Normal Tissue, show that the finding of a nucleoprotein (ribose type) is not evidence of a virus since both mouse milk containing the milk factor and normal tissues contain nucleoproteins of the same type.

A complete survey on the Inciting Influences in the Etiology of Mammary Cancer in Mice is given by J. J. Bittner. The three inciting influences (the inherited susceptibility, the hormonal stimulation, and the agent transferred in the mother's milk) which have been recognized as causative factors in the production of spontaneous mammary carcinoma in mice are described in detail.

Another paper on the Properties and Nature of the Milk Agent in the Genesis of Mammary Tumors in Mice is written by M. B. Shimken and H. B. Andervont. In this paper which deals with the biologic data, it is shown that the agent is positive in action and also on some of its physical and chemical properties.

A short note on Nonhereditary Nursing Influence in Leukemia by J. Furth follows.

The next series of papers deal with carcinogenesis, and the first, Hydrocarbon Carcinogenesis by L. F. Fieser to whom cancer research owes much, deals with the correlation between structure and activity of the carcinogenic hydrocarbons. The unusual reactivity of these carcinogens with other chemicals in vitro is discussed, and from these characteristics of

the hydrocarbons several hypotheses are presented as possible explanations for the initiation of cancer by the reactivity of these compounds.

In the Relationship of Pyrrol Compounds to Carcinogenesis, presented by F. H. J. Figge, are discussed three hypothetical factors (Factor I, Carcinogenic Steroidal Hydrocarbons or Other Compounds; Factor II, Metabolizing or Oxidizing Agents; and Factor III, Sensitizing Agents) which interact to produce cancer. The porphyrins hypothetically act as sensitizers to carcinogenic stimuli.

The next article deals with the Time and Site of Origin of the Leukemic Cell by J. Furth and M. C. Boon. This study deals with the time of origin (during young adult life and shortly before the onset of malignancy) and the site, which either may be in numerous foci or in one or a few foci, of the leukemic cell in Ak mice.

A summary of Certain Data on the Production of Malignancy in Vitro by W. R. Earle presents the mode of action of methylcholanthrene on connective tissue fibroblasts grown in vitro in a completely heterologous medium for five years. Characteristics of the untreated and treated fibroblasts with respect to the average increase in the width of the cultures, percentage of tumors produced after inoculation in C3H mice, rate of tumor growth in the latter, anaerobic glycolysis, distally located Golgi material, and percentage of vascular tissue elicited upon transplantation are discussed with relationship to the effect of the carcinogen upon the fibroblasts.

The next article is the Occurrence of Dependent and Autonomous Phases in the Development of Cancer by H. S. N. Greene. In experiments using his well-known technique of transplanting tumors to the anterior chamber of the eye, the author postulates that a cancer is not malignant unless it will grow heterologously in that organ.

A Possible Common Mitochondrial Origin of the Variegational and Virus Diseases in Plants and Cancer in Animals is then presented by H. G. Du Buy and M. W. Woods. This interesting and thought-provoking paper deals with the variegational diseases of plants in which alterations in the mitochrondria or plastids can be followed by metabolic and cytological changes in a specific cytoplasmic particulate. The investigations of these authors suggest that viruses may be derived by gradual alterations in normally occurring cytoplasmic components, the mitochrondria. Once the derived mitochrondria or plastial is formed, it is self-perpetuating and moreover it contains a ribose nucleoprotein, which is characteristic of animal viruses.

The first paper on enzymes, Biochemical Studies of Chemical Carcinogenesis, With a Preliminary Note on the Susceptibility to Chemicals of Certain Neoplasms, both Animal and Human, by C. J. Kensler and C. P. Rhoads, describes the biochemistry of p-dimethylaminoazobenzene, which produces tumors of the liver in the rat. The results of the fine experiments on the inhibitory effect of the metabolites of p-dimethylaminoazobenzene upon a diphosphopyridine nucleotide from yeast, which ferments glucose, cocarboxylase-carboxylase system, upon the respiration of liver slices, upon the oxygen consumption of liver brei, and upon succinic dehydrogenase and cytochrome oxidase are of great value for an understanding of the process of carcinogenesis. A portion of the paper deals with the correlation of inhibitory effects of the split products of methyl derivatives of aminoazobenzene on enzyme systems and cellular respiration with the carcinogenic potency of the parent azo molecule.

The contribution by J. P. Greenstein on Enzymes in Normal and Neoplastic Animal Tissues is a composite picture of the authors excellent investigations on enzyme patterns in normal and neoplastic tissues. Using a number of enzymatic activities of normal mouse tissues and neoplastic tissues of the mouse, six generalizations are made comparing the enzyme activities of both groups of tissues. The generalizations serve to characterize more exactly the biochemical nature of normal and malignant growths. In a similar manner the author has investigated the enzyme activities of the liver of the rat and of the normal adult, regenerating, fetal, and hepatoma and has found that enzymatic patterns of the hepatoma and of the fetal liver resemble each other more than do the similar normal adult and regenerating liver. Normal and neoplastic human tissues are also placed into categories depending upon the response of the tissue suspensions to p-phenylenediamine in the presence of an excess of cytochrome C.

An abstract of Distribution of Enzymatic Activities in Fractions in Mammalian Liver by A. Claude describes the distribution of cytoplasmic enzymes associated with the free secretory granules, the mitochondria, the microsome fraction, and the supernate.

The Serology of Cathepsins by M. E. Maver and J. W. Thompson is a study on the cathepsins, which are endocellular proteinases responsible for the synthesis and lysis of tissue proteins, and is obviously of great importance in growth phenomena.

Reducing Properties of Serum From Malignant and Nonmalignant Patients and From Normal Individuals by R. J. Savignac, J. C. Gant, and I. W. Sizer is the next presentation in which the time to reduce methylene blue by the serum of normal, noncancerous and cancerous patients is discussed. The reducing activity was found to be located in the albumin fraction and is due to the liberation of sulfhydryl groups by the action of sodium hydroxide.

In the first paper on diet and cancer, the article B Vitamins and Cancer by R. J. Williams, the author describes the investigations in his laboratory on the content of the B vitamins in human and rat normal tissues and in human and rat cancers.

H. P. Rusch, C. A. Baumann, J. A. Miller, and B. E. Kline then describe their studies on Experimental Liver Tumors. The production of tumors on purified diets and the effect of riboflavin and casein, biotin, pyridoxine, and lipids on the genesis of hepatic tumors is described.

A note on Some Aspects of Diet and Cancer by A. Tannebaum follows in which the author stresses the importance of diet in experimental investigations in cancer of mice. His contributions to this phase of cancer research should be carefully read by all in this field of endeavor.

The Effects of Therapeutic Agents on Human and Mouse Leukemia by C. M. Flory is the first of five papers on Chemotherapy of Cancer. In the first paper the effect of many compounds on mouse and human leukemia were tested, and the agents were at best either only palliative or ineffective.

The next article is on the Effect of Various Agents on Normal and Malignant Tissues by H. O. Singher, C. J. Kensler, and C. P. Rhoads. The Warburg method and the egg technique are used to test the effectiveness of various compounds upon normal and malignant tissues.

The results by G. O. Gey, M. K. Gey, F. Inui, and H. Vedder on Penicillin Action on Strains of Normal and Cancer Cells demonstrate no evidence of an increased tumor cell susceptibility by sodium penicillin as compared to normal cells from the results obtained on continuous cultures and on primary explants of tumors produced by inoculations of continuous cultures of tumor cells. The final notes also deal with the influence of penicillin on tumor cultures or in tumor tissue.

CHRISTOPHER CARRUTHERS, M.D.